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(54) Title: BIOLOGICALLY ACTIVE FRAGMENTS OF THERMUS FLAVUS DNA POLYMERASE

(57) Abstract

The present invention is directed to a DNA encoding a biologically active fragment of a thermostable, full length DNA polymerase I enzyme of *Thermus flavus*. More particularly, the invention is directed to a DNA encoding an approximately 63,000 dalton DNA polymerase that lacks 274 amino acids from the N-terminus of the approximately 94,000 dalton *T. flavus* DNA polymerase I, and to the protein encoded thereby which has been designated the *T. flavus* DNA polymerase I exo fragment. The enzyme fragments are useful in DNA sequencing, Thermal Cycle Labeling, Polymerase Chain Reaction, and other molecular biological applications.

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BIOLOGICALLY ACTIVE FRAGMENTS OF *THERMUS FLAVUS* DNA POLYMERASE

BACKGROUND OF THE INVENTION

A. Field of the Invention

The present invention relates to an isolated and purified DNA that encodes a thermostable DNA polymerase. Additionally, the present invention relates to a recombinant and thermostable DNA polymerase and to fragments thereof, all having enhanced polymerase activity, and to methods for producing the DNA polymerase and fragments. The present invention further relates to recombinant fragments having decreased exonuclease activity. The thermostable recombinant polymerases of the present invention are useful because they are capable of providing enhanced polymerase activity in bio-applications, such as in the polymerase chain reaction (PCR), in DNA amplification and in thermal cycle labeling (TCL).

B. Background

The burgeoning field of biotechnology was revolutionized by recombinant DNA technology, and DNA polymerase enzymes are an indispensable tool used in many modern molecular *in vitro* recombinant DNA biological applications, such as in DNA sequencing; DNA cycle sequencing; Polymerase Chain Reaction (PCR) and its many variations (see, e.g., Erlich *et al.*, Current Communications in Molecular Biology: Polymerase Chain Reaction. Cold Spring Harbor Press, Cold Spring Harbor (1989); Innis *et al.*, PCR protocols: A guide to methods and applications. Academic Press, San Diego (1990)); Thermal Cycle Labeling (TCL) (Mead and Swaminathan, U.S. Patent App. Ser. No. 08/217,459, filed March 24, 1994; PCT App. No. US94\03246, filed March 24, 1994); Random Primer Labeling (RPL); Ligase Chain Reaction (LCR) (Wiedmann et al., *PCR Methods and Applications 3*: S51-S64 (1994)); and other applications.

To date scientists have reported more than 40 different DNA polymerases, and have reported DNA sequence information for some DNA polymerase genes. Amino acid sequence information has been deduced from the reported genes, and comparison of amino acid sequences has resulted in the placement of reported polymerase genes into four major families: namely, A, B, C, and X. Family A contains *E. coli* DNA polymerase I, an enzyme involved in repair of DNA and in replication during fast growth. Family B includes *E. coli* DNA polymerase II. Family C polymerases include *E. coli* DNA polymerase III, the major replication enzyme. The fourth group, Family X, contains enzymes such as the eukaryotic DNA polymerase β and eukaryotic terminal transferases (Ito and Braithwaite, *Nucleic Acids Res. 19*: 4045-4057 (1991)). The breakdown of DNA polymerases into families has been helpful for the understanding of fundamental biological processes and for the selection of enzymes for particular molecular biological applications.

DNA polymerase I (pol I) (Family A) enzymes have proved to be very useful for DNA sequencing applications, PCR, TCL, and other applications known in the art. Structure-function relationship studies indicate that known DNA pol I molecules share a similar modular organization. A 5′ → 3′ exonuclease function is located in the N-terminal one-third of the enzyme. The remainder of the molecule forms one domain which is further classified into functional sub-domains. Adjacent to the 5′ → 3′ exonuclease domain lies a 3′ → 5′ exonuclease sub-domain, followed by a polymerase sub-domain (Blanco et al., *Gene 100*:27-38 (1991)).

In addition to classifying DNA polymerase enzymes into the above families, it is also useful to classify such polymerases as mesophilic (purified from mesophilic organisms) or thermophilic (purified from thermophilic organisms) in origin. DNA polymerases of mesophilic organisms were discovered earlier and have been more extensively studied than their thermophilic counterparts. As early as the 1950's, isolation and purification protocols for DNA polymerase I from mesophilic bacteria (e.g.,

E. coli) and some of their phages were developed and have since been modified. See, e.g., Bessman *et al.*, *J. Biol. Chem. 233*:171-177 (1958); Buttin and Kornberg, *J. Biol. Chem. 241*:5419-5427 (1966). The DNA polymerases studied most extensively are the DNA polymerase I enzymes isolated from *E. coli* and the bacteriophage T7 DNA polymerase.

The DNA polymerases of mesophilic origin are useful in many biological applications, such as in certain DNA sequencing applications. However, many important applications (e.g., polymerase chain reaction (PCR) applications and thermal cycle labeling (TCL)) require thermal cycling to repeatedly denature template DNA and/or RNA and their extension products. Because mesophilic DNA polymerases do not withstand the high temperatures or thermal cycling of these applications, thermostable DNA polymerases enjoy significant advantages over mesophilic DNA polymerases in such applications.

The discovery and study of such thermostable DNA polymerases -- from thermophilic bacteria -- has been a much more recent phenomenon. See, e.g., Uemori et al., *J. Biochem. 113:* 401-410 (1993); Uemori et al., *Nucleic Acids Res. 21:* 259-265 (1993)); Lawyer et al. *J. Biol. Chem. 264:* 6427-6437 (1989); Kaledin et al., *Biokhimiya 45*:644-651 (1980); Chien et al., *J. Bacteriol. 127*:1550-1557 (1976); Gelfand *et al.*, U.S. Patent Nos. 4,889,818 and 5,079,352; Burke et al., U.S. Patent No. 5,108,892.

Perhaps the best-studied thermostable DNA polymerase, derived from *Thermus aquaticus*, is called Taq pol I. A number of routes have been taken in attempts to clone the Taq DNA pol I gene. (See, e.g., Lawyer *et al.* (1989); Gelfand *et al.*, U.S. Patent No. 5,079,352 (1992) (purification to approx. 200,000 units/mg reported); Lawyer *et al.*, *PCR Methods and Applications 2*:275-287 (1993) (purification to 292,000 units/mg reported); Engelke *et al.*, *Anal. Biochem. 191*:396-400 (1990); Sagner et al., *Gene 97*:119-123 (1991)).

As explained above, in addition to possessing useful DNA polymerase activity, a number of DNA polymerase I holoenzymes possess exonuclease activities, which for many biological applications are undesirable. Therefore, modified DNA polymerase enzymes having reduced exonuclease activities are desirable. Through deletion of the 5′ one-third of DNA polymerase I genes, or the proteolytic cleavage and subsequent removal of the portion of the holoenzyme encoded thereby, scientists have created DNA pol I fragments retaining polymerizing activity, but having reduced 5′ → 3′ exonuclease activity. (See, e.g., Joyce and Grindley, *Proc. Natl. Acad. Sci.* *80*:1830-1834 (1983) (the Klenow-Fragment of the *E. coli* DNA polymerase enzyme); Lawyer *et al.*, *J. Biol. Chem. 264*:6427-6437 (1989), Gelfand *et al.*, U.S. Patent No. 5,079,352 (1992), Lawyer *et al.*, *PCR Methods and Applications* 2:275-287 (1993) (the Stoffel fragment of the *T. aquaticus* (Taq) DNA polymerase enzyme, reportedly purified to a specific activity of 369,000 units/mg); and Barnes, *Gene 112*:29-35 (1992) (the KlenTaq DNA polymerase).)

In addition to Taq DNA polymerases, other thermophilic DNA polymerases reportedly have been cloned and expressed in *E. coli*. Uemori *et al.* reportedly expressed DNA polymerases from *Bacillus caldotenax* (*J. Biochem. 113*:401-410 (1993)) and *Pyrococcus furiosus* (*Nucleic Acids Res. 21*:259-265 (1993)).

DNA polymerases from other bacteria of the genus *Thermus* have been reported. A method of recovering a thermostable DNA polymerase from cultured *Thermus thermophilus* is reported in U.S. Patent No. 5,242,818 to Oshima et al. (1993). The purported purification of native *Thermus flavus* DNA polymerase with an apparent molecular weight of 66,000 daltons was described by Kaledin *et al.*, *Biokhimiya 46*:1576-1584 (1981)). In one application, Kainz *et al.*, *Anal. Biochem. 202*:46-49 (1992), reported the amplification of a 10.9 kb fragment and a 15.6 kb fragment from phage

lambda DNA with Hot Tub *(T. flavus)* polymerase (Amersham, Arlington Heights, IL), but not with Taq polymerases. The rapid filter assay of Sagner et al., *Gene 97*:119-123 (1991) has allowed Akhmetzjanov and Vakhitov to identify a purported *T. flavus* (strain and origin unidentified) DNA polymerase I gene and to determine the DNA sequence of this gene (*Nucleic Acids Res. 20*:5839 (1992)). There is no report of the expression of an active DNA polymerase encoded by the purported *Thermus flavus* DNA polymerase I (Tfl DNA pol I) gene characterized by Akhmetzjanov and Vakhitov. Native *T. flavus* (Tfl) DNA polymerase I is commercially available, e.g., from Molecular Biology Resources, Inc. (Milwaukee, WI, Catalogue #1112-01).

The different reports of thermostable DNA polymerases and their derivatives suggest these enzymes possess different, unpredictable properties that may be advantageous or detrimental, depending on the biological application in which the DNA polymerase is to be employed. For example, *Thermus thermophilus* DNA polymerase I was reported to have a significant reverse transcriptase activity. In the same reaction tube, in successive steps, the reverse transcriptase function allows the production of double stranded DNA from RNA and then the DNA polymerase function is used to amplify this cDNA. Myers and Gelfand, *Biochemistry 30*:7661-7666 (1991).

The KlenTaq DNA polymerase is an example of an enzyme fragment with important properties differing from the Taq holoenzyme. The KlenTaq DNA polymerase reportedly has a roughly two-fold lower PCR-induced relative mutation rate than Taq polymerase holoenzyme. However, more units of KlenTaq are needed to obtain PCR products similar to those generated with Taq DNA pol I.

Similarly, Lawyer et al. (1993) reported that *T. aquaticus* DNA polymerase I fragments possessed greater thermostability and were active over a broader Mg^{++} -range than the corresponding holoenzyme. Because of its

broader range of magnesium ion concentration, the Stoffel fragment has been used in multiplex PCR, where more than two primers must anneal to the template. The thermostability of the Stoffel fragment makes this enzyme a better choice when GC-rich templates are amplified. It is desirable to purify and isolate additional DNA polymerase enzymes and derivatives, to take advantage of the unique but unpredictable properties that such molecules may have.

There remains a need in the art for new, thermostable DNA polymerase enzymes for use in the expanding universe of molecular biological applications. More particularly, there exists a need for thermostable DNA polymerase enzymes having high purity, high DNA polymerase specific activity, low levels of exonuclease activity, and possessing high fidelity (low mutation frequencies) and high processivity when used in DNA amplification protocols.

An object of the present invention is to provide polymerase enzyme preparations of greater purity, quantity, DNA polymerase specific activity, and processivity than has heretofore been possible. A further object is to eliminate the need and expense of culturing of large volumes of thermophilic bacteria at high temperatures that is associated with preparing thermostable polymerase enzyme preparations. Yet another object is to provide a recombinant polymerase possessing reduced exonuclease activities, as compared to the currently available native holoenzyme.

SUMMARY OF THE INVENTION

The present invention relates to the cloning and expression of a gene encoding a thermostable DNA polymerase, the purification of a recombinant thermostable DNA polymerase encoded by the gene, and applications for using the polymerase. The gene of the *Thermus flavus* DNA polymerase I (Tfl DNA pol I), was cloned and expressed in *Escherichia coli*. The purified recombinant *T. flavus* DNA polymerase enzyme is shown to be

thermostable and have a molecular weight of about 90,000 to 100,000 daltons. The DNA sequence of the Tfl DNA pol I gene, including flanking sequences, was determined and the coding sequence of the recombinant enzyme was mapped within this gene. A Tfl DNA Pol I gene fragment also was expressed in *E. coli*, the purified recombinant protein products ("exo⁻ fragment") lacking 274 and 275 amino acids from the N-terminus of the Tfl DNA pol I holoenzyme. This Tfl exo⁻ fragment has very low 3′ → 5′ and 5′ → 3′ exonuclease activities. Numerous properties of and applications for the recombinant enzymes are described.

In one aspect, this invention provides purified polynucleotides (e.g. DNA sequences and RNA transcripts thereof) encoding a thermostable polypeptide having DNA polymerase activity. Preferred DNAs include the *Thermus flavus* DNA pol I gene comprising nucleotides 301 to 2802 of SEQ ID NO: 1; the *Thermus flavus* DNA pol I exo⁻ fragment gene comprising nucleotides 1 to 1791 of SEQ ID NO: 3; the DNA comprising nucleotides 112-1791 of SEQ ID NO: 3; a portion of the insert of plasmid pTFLRT4 (ATCC Accession No. 69633), said portion encoding a thermostable polypeptide having DNA polymerase activity; a portion of the insert of plasmid p21EHcM1.1, (ATCC Accession No. 69632), said portion encoding a thermostable polypeptide having DNA polymerase activity; fragments or portions of these DNAs that encode thermostable polypeptides having DNA polymerase activity; and variants of these DNAs that encode thermostable polypeptides having DNA polymerase activity.

In another aspect, this invention provides DNA sequences such as those described above operatively linked to a promoter sequence, a cloning vector, an expression vector, or combinations thereof.

In related aspects, the invention provides novel plasmids and vectors. For example, the invention provides a plasmid pTFLRT4, having ATCC Accession No. 69633; and a plasmid p21EHcM1.1, having ATCC Accession No. 69632. The invention also provides a vector that includes

nucleotides 301 to 2802 of SEQ ID NO:1, the nucleotides encoding a polypeptide having thermostable DNA polymerase activity; and a vector that includes nucleotides 112 to 1791 of SEQ ID No:3, the nucleotides encoding a polypeptide having thermostable DNA polymerase activity.

In related aspects, the invention provides a vector having at least one insert consisting essentially of nucleotides 301 to 2802 of SEQ ID NO: 1, the nucleotides encoding a polypeptide having thermostable DNA polymerase activity. The invention further provides a vector having at least one insert consisting essentially of nucleotides 112 to 1791 of SEQ ID NO: 3, the nucleotides encoding a polypeptide having thermostable DNA polymerase activity.

The present invention is also directed to host cells, such as prokaryotic and eukaryotic cells, that have been stably transformed with DNAs vectors, or plasmids of the invention. Another aspect of the invention is directed to such transformed host cells that are capable of expressing a thermostable polypeptide encoded by the DNAs, the peptide having DNA polymerase activity.

In another aspect, this invention provides purified thermostable polypeptides having DNA polymerase activity. Preferred peptides include a *Thermus flavus* DNA polymerase I holoenzyme substantially free of other *Thermus flavus* proteins; a polypeptide having the amino acid sequence of SEQ ID NO: 2; a fragment of a *Thermus flavus* DNA polymerase I holoenzyme, including a fragment with reduced exonuclease activity as compared to the holoenzyme, and also including a fragment having the amino acid residues 1-560 or 2-560 of the amino acid sequence shown in SEQ ID NO: 5; a fragment encoded by the insert of plasmid p21EHcM1.1, having ATCC Accession No. 69632; fragments of the above peptides that retain DNA polymerase activity; and variants of the above peptides that retain DNA polymerase activity.

In another aspect, this invention provides methods for purifying a thermostable polypeptide having DNA polymerase activity including the steps of transforming a host cell with a DNA of the present invention to create a transformed host cell; cultivating the transformed host cell under conditions that promote expression of a thermostable polypeptide encoded by the DNA, the polypeptide having DNA polymerase activity; and purifying the thermostable polypeptide with a monoclonal antibody that is cross-reactive with the thermostable polypeptide. In one preferred method, the cross-reactive monoclonal antibody has specificity for a *Thermus aquaticus* DNA polymerase and/or for a *Thermus flavus* DNA polymerase.

In another preferred method, commercially available chromatography columns are used to purify the expressed polypeptide.

In another aspect, this invention provides methods of purifying a thermostable polypeptide having DNA polymerase activity. One such method includes the steps of expressing the thermostable polypeptide in a host cell, the polypeptide having an amino acid sequence encoded by a DNA of the present invention; lysing the cell to create a suspension containing the thermostable polypeptide, as well as host cell proteins and cell debris; contacting a soluble portion of the suspension with an antibody that is immunologically cross-reactive with the thermostable polypeptide under conditions wherein the antibody binds to the thermostable polypeptide to form an antibody-polypeptide complex; isolating the antibody-polypeptide complex; and separating the thermostable polypeptide from the isolated antibody-polypeptide complex to provide a purified thermostable polypeptide. Preferably, such a method further includes the steps of heating the suspension to denature the host cell proteins; and centrifuging the suspension to remove the cell debris and denatured host cell proteins. In more preferred methods, the immunologically cross-reactive antibody is a monoclonal antibody, such as a monoclonal antibody that is immunologically cross-reactive with *Thermus aquaticus* DNA polymerase I and/or *Thermus flavus* DNA polymerase I. This

preferred method is exemplified herein using the monoclonal antibody purified from a hybridoma designated hybridoma 7B12.

In another aspect, this invention provides methods of using the DNA constructs of the invention to produce recombinant thermostable polypeptides having DNA polymerase activity. One such method involves using a DNA encoding a DNA polymerase enzyme to generate active fragments of the DNA polymerase enzyme, including the steps of: deleting a portion of the DNA to create a modified DNA; expressing the modified DNA to produce a DNA polymerase enzyme fragment; purifying the DNA polymerase enzyme fragment; assaying the DNA polymerase enzyme fragment for DNA polymerase activity; and selecting a DNA polymerase enzyme fragment having DNA polymerase activity; wherein the DNA is selected from among the DNAs described herein.

In another aspect, this invention provides methods for using the proteins of the invention in biological applications, such as DNA sequencing; amplification of DNA and/or RNA sequences; polymerase chain reaction (PCR); thermal cycle labeling (TCL); universal thermal cycle labeling (UTCL); ligase chain reaction (LCR); and other applications or processes that would be apparent to those skilled in the art.

In yet another aspect, this invention provides kits for using the proteins of the invention in various biological applications, such as kits for labeling DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES **1A** and **1B** graphically depict the cloning strategy: (1A) for the gene encoding the Tfl DNA pol I holoenzyme; and (1B) for the DNA encoding the exo⁻ fragment of *T. flavus* DNA polymerase I. The abbreviations used are: B: BamHI, RI: EcoRI, RV: EcoRV, Hc: HincII, P lacZ: promoter of the lacZ gene, S: SalI, and X: XbaI. Jagged lines (≈≈ ≈≈) represent vector DNA; straight horizontal (———) lines represent Tfl

insert DNA; dark and light shaded rectangles depict Tfl DNA pol I gene sequences. The graphical depictions are not drawn to scale, and not all available restriction sites are shown in all steps.

FIGURE 2 depicts the DNA sequence and the deduced amino acid sequence for the Tfl DNA pol I holoenzyme coding sequence and for 5′ untranslated and 3′ untranslated sequences. The circled amino acid (Glu_{239}) is the first amino acid believed to be translated during translation of plasmid p21EHcM1.1, encoding the Tfl exo⁻ fragment. The boxed amino acid (Leu_{275}) is the amino acid determined to be the first amino acid of the purified and isolated major Tfl exo⁻ fragment. An asterisk (*) indicates the stop codon TAG.

FIGURE 3 is a comparison of deduced amino acid sequences from the *Thermus flavus* DNA polymerase I of this invention (MBR TFL); *Thermus aquaticus* DNA polymerase I (TAQ) reported in Lawyer *et al.*, *J. Biol. Chem.* 264:6427-6437 (1989)); and purported *Thermus flavus* DNA polymerase I (A&V TFL) described in Akhmetzjanov and Vakhitov, *Nucleic Acids Res.* *20*:5839 (1992). The sequences were aligned to maximize homology. Conservative differences between the amino acid sequences are indicated with asterisks (*) and non-conservative differences are indicated with arrowheads (^).

FIGURE 4 depicts double-stranded DNA sequence of the *T. flavus* DNA pol I gene, including 5' untranslated and 3' untranslated sequences. Lower case letters indicate untranslated sequences, upper case letters represent the coding sequence. The start codon (ATG) of Tfl DNA pol I is at positions 301-303, and the stop codon is at positions 2803-2805. The positions in the sequence that correspond to synthetic primers used in sequencing reactions have been indicated with boxes. The sequence of the 2-4 fragment is underlined with an arrow (<————————>).

FIGURE 5 depicts the relative DNA polymerase enzymatic activity, at different buffered pH levels, of native *Thermus flavus* holoenzyme (nTfl Holo: empty squares); recombinant *Thermus flavus* holoenzyme (rTfl Holo: diamonds); *Thermus flavus* exo⁻ fragment (Tfl exo⁻: circles); *T. aquaticus* DNA pol I (AmpliTaq: crossed boxes); and the Taq enzyme Stoffel fragment (Stoffel: triangles).

FIGURE **6A** depicts the relative DNA polymerase enzymatic activity, at different concentrations of $MgCl_2$, of native *Thermus flavus* holoenzyme (nTfl Holo: empty squares); recombinant *Thermus flavus* holoenzyme (rTfl Holo: diamonds); *Thermus flavus* exo⁻ fragment (Tfl exo⁻: circles); and *T. aquaticus* DNA pol I Stoffel fragment (Stoffel: triangles).

FIGURE **6B** depicts the relative DNA polymerase enzymatic activity, at different concentrations of $MnCl_2$, of native *Thermus flavus* holoenzyme (nTfl Holo: empty squares); recombinant *Thermus flavus* holoenzyme (rTfl Holo: diamonds); *Thermus flavus* exo⁻ fragment (Tfl exo⁻: circles); and *T. aquaticus* DNA pol I Stoffel fragment (Stoffel: triangles).

FIGURE **7A** depicts the relative DNA polymerase enzymatic activity, at different temperatures, of native *Thermus flavus* holoenzyme (nTfl Holo: open boxes); recombinant *Thermus flavus* holoenzyme (rTfl Holo: diamonds); *Thermus flavus* exo⁻ fragment (Tfl exo⁻: circles); and *T. aquaticus* DNA pol I Stoffel fragment (Stoffel: triangles).

FIGURE **7B** photographically depicts the relative quantities of PCR amplification product generated after 25, 30, and 35 reaction cycles, using 10 units of Tfl exo⁻ fragment (E) or Stoffel fragment (S) as the PCR DNA polymerase. The far right lane depicts the PCR amplification product generated after 35 reaction cycles using 1.1 unit of Tfl exo⁻ fragment.

FIGURE **8** depicts enzymatic stability in thermal cycling (relative DNA polymerase enzymatic activity after different numbers of PCR cycles), of native *Thermus flavus* holoenzyme (nTfl Holo: empty squares);

Thermus flavus exo⁻ fragment (Tfl exo⁻; circles); *T. aquaticus* DNA pol I (AmpliTaq: crossed squares); and *T. aquaticus* DNA pol I Stoffel fragment (Stoffel: triangles).

FIGURE **9** photographically depicts the purity of purified *E. coli* DNA polymerase I (Eco Pol I, control), recombinant *Thermus flavus* holoenzyme (rTfl Holo), and *Thermus flavus* exo⁻ fragment (Tfl exo⁻) on a 12.5% SDS-PAGE gel stained with silver.

FIGURES **10A**, **10B**, **10C**, and **10D** photographically depict portions autoradiographs of sequencing gels showing DNA sequence obtained with the indicated polymerases substituted into the SEQUAL™ or the Cycle SEQUAL™ DNA Sequencing Kit. Abbreviations: recombinant *Thermus flavus* holoenzyme (Tfl Holo); *Thermus flavus* exo⁻ fragment (Tfl exo⁻); *T. aquaticus* DNA pol I holoenzyme (AmpliTaq); and the Taq enzyme Stoffel fragment (Taq Stoffel).

DETAILED DESCRIPTION OF THE INVENTION

This application describes the isolation and characterization of the gene coding for *Thermus flavus* (ATCC Accession No. 33923) DNA polymerase I (Tfl DNA pol I) and having homology to the family A enzymes described above. Also described is the expression of this gene in *E. coli* and the purification and characterization of the recombinant DNA polymerase. The cloning and expression of an active fragment of the *Thermus flavus* DNA polymerase gene is also described, and the gene fragment and expressed peptides are characterized. Recombinant vectors and host cells are also described. Additionally, methods and kits are described that involve the DNAs and proteins of the present invention. Thus, as the discussion below details, the present invention has several aspects.

As a first step in the generation of the DNAs and polypeptides of the present invention, native *T. flavus* DNA polymerase I was purified and

isolated from *T. flavus* cells (ATCC Accession No. 33923) and digested with trypsin, and amino acid sequence information was obtained from one of the reaction products (i.e. from a trypsin digest protein fragment). (See Example 1.) Additionally, a *Thermus flavus* genomic library was constructed in phage λ Dash II and amplified. (See Example 2.)

The amino acid sequence information generated in Example 1, together with published amino acid sequence information from the *Thermus aquaticus* DNA pol I gene, was used to create synthetic DNA primers for isolating a portion of the *Thermus flavus* DNA polymerase I gene. (Example 3.) More particularly, a first primer, designated FTFL2, was synthesized to correspond with known coding sequence from *T. aquaticus* DNA pol I gene (Lawyer *et al.*, *J. Biol. Chem 264*: 6427-6437 (1989)), and to bind to the top strand of the *T. aquaticus* DNA pol I gene. The particular *T. aquaticus* coding sequence chosen encodes a portion of the *T. aquaticus* DNA pol I amino acid sequence that is homologous to the native *T. flavus* DNA pol I peptide that had previously been sequenced (Example 1). A second primer, designated RTFL4, was synthesized to have a sequence that binds to the 3´-end of the *T. aquaticus* gene on the opposite strand. A DNA amplification reaction was performed with primer FTFL2, primer RTFL4, and *T. flavus* genomic DNA. The amplification reaction yielded a single amplification product, designated the "2-4 fragment." This fragment was cloned into M13mp18 vector, amplified in *E. coli*, and sequenced.

As explained in detail in Example 4, the 2-4 fragment (obtained by the procedures outlined in Example 3) was used to isolate the *Thermus flavus* DNA pol I gene from the *T. flavus* genomic library that had been constructed (Example 2). Specifically, the 2-4 fragment was further amplified and used to generate probes via thermal cycle labeling (TCL). The amplified *T. flavus* genomic library was plated on 2XTY plates and grown until plaques formed. Duplicate plaque lifts were obtained from each plate onto Hybond

N filters, and these filters were then screened using the above-described TCL probes using hybridization methods well known in the art. Positive plaques were selected, purified by dilution and by re-screening with the 2-4 probes, and then further characterized. In particular, two clones with inserts of 14-16 kb, designated λ21 and λ51, were chosen for further analyses.

Clones λ21 and λ51 were used as a starting point from which the complete *T. flavus* DNA pol I gene was cloned and sequenced. As explained in detail in Example 5 and with reference to FIGURE 1A, restriction mapping, subcloning, and partial sequencing led to the determination that a subclone of λ21 designated p21E10 contained about 2/3 of the Tfl DNA pol I gene (3' end), whereas a subclone from λ51 designated p51E9 contained a 5' portion of the gene that overlapped the coding sequence contained in clone p21E10.

A primer walking procedure was used to obtain the complete sequence of the gene. Specifically, primers homologous or complimentary to the ends of previously determined sequences (obtained from p21E10 and from other deletion vectors) were synthesized and used in additional sequencing reactions. By repeating this process the entire length of the gene was sequentially sequenced. The DNA and deduced amino acid sequence for the *T. flavus* DNA pol I holoenzyme are shown in FIGURE 2, which corresponds to SEQ. ID NO: 1 and 2 in the Sequence Listing. The sequences of each primer used, and the relative location of the primers in the gene sequence, are depicted in Table 2 and in FIGURE 4, respectively. The amino acid sequence of the holoenzyme depicted in FIGURE 2 and SEQ. ID NO: 2 corresponds with nucleotides 301 to 2802 of the DNA depicted in FIGURE 2 and SEQ ID NO: 1.

The foregoing results demonstrate that an aspect of the invention is directed to a purified DNA encoding a thermostable polypeptide having DNA polymerase activity, the DNA comprising nucleotides 301 to 2802 of SEQ ID NO: 1. This DNA may be operatively linked to other

DNAs, such as expression vectors known in the art. The invention is also directed to a vector having at least one insert consisting essentially of nucleotides 301 to 2802 of SEQ ID NO: 1, the nucleotides encoding a thermostable polypeptide having DNA polymerase activity. Similarly, the invention is directed to a vector comprising nucleotides 301 to 2802 of SEQ ID NO:1, the nucleotides encoding a polypeptide having thermostable DNA polymerase activity.

With the gene sequence established, the DNA and deduced amino acid sequences of the *T. flavus* DNA pol I gene were aligned and compared to the DNA and deduced amino acid sequences of the purported Tfl DNA pol I published by Akhmetzjanov and Vakhitov, *Nucleic Acids Res. 20*:5839 (1992) (83% DNA sequence homology, 85% amino acid sequence homology) and to the deduced amino acid sequence of the Taq pol I gene (86% DNA sequence homology, 87% amino acid sequence homology). The amino acid comparison is depicted in FIGURE 3.

To produce a recombinant *T. flavus* DNA pol I protein a full-length *T. flavus* DNA pol I gene clone was constructed, expressed in *E. coli*, and purified. As detailed in Example 6 and FIGURE 1A, plasmids p51E9 and p21E10 were further restriction mapped and subsequently subcloned to generate plasmid p21BRV2, containing a 1.3 kb insert that includes the 3' region of the Tfl DNA pol I gene, and plasmid p51X16, containing a 2.5 kb BamHI fragment in which the 5' region of the gene was located. Linearization of plasmid p21BVR2 with BamHI and ligation of this linearized plasmid to the BamHI fragment of p51X16 yielded clone pTFL 1.4, containing the entire Tfl DNA pol I gene.

E. coli DH5αF' were transformed with plasmid pTFL 1.4 and grown in a fermentor to recombinantly produce *T. flavus* DNA pol I holoenzyme. As detailed in Example 6, this recombinant protein was purified from the lysed *E. coli* with a method that included a heat denaturation of *E.*

coli proteins, precipitations and centrifugations, Sephadex G-25 and Bio-Rex 70 column chromatography, and immunoaffinity chromatography. The calculated DNA polymerase specific activity of *T. flavus* DNA pol I isolated by this procedure was determined to be 79,500 U/mg protein.

In order to increase the yield of recombinant *T. flavus* DNA pol I holoenzyme, a second expression clone was constructed in which the lacZ promoter was fused directly to the initiation codon of the Tfl DNA pol I gene. As detailed in Example 7 and FIGURE 1A, the promoter was fused to the 5' portion of the gene located using site-directed mutagenesis. and a second generation expression clone, designated pTFLRT4, was generated.

E. coli (strain DH5αF'IQ) were transformed with pTFLRT4 and cultivated, and recombinant *T. flavus* DNA pol I was isolated therefrom and purified. As detailed in Example 7, the purification protocol includes heat treatment, polyethyleneimine- (PEI-) precipitation, $(NH_4)_2SO_4$- precipitation, Bio Rex 70 chromatography and immunoaffinity chromatography. The yield was approximately 2,000,000 units of enzyme from 500 g of cells, and the purified enzyme preparation was found to have a DNA polymerase specific activity of 217,600 U/mg protein. The N-terminal amino acid sequence of the recombinant Tfl DNA pol I enzyme was determined and found to be identical to the sequence deduced from the *T. flavus* DNA Pol I gene sequence.

The foregoing discussion demonstrates that an aspect of the invention is directed to a purified DNA comprising a portion of the insert of plasmid pTFLRT4, the plasmid having ATCC Accession No. 69633, the portion encoding a thermostable polypeptide having DNA polymerase activity. This DNA may be operatively linked to additional DNAs, such as promoter DNAs and/or expression vector DNAs known in the art. A preferred DNA is plasmid pTFLRT4 itself. The present invention is also directed to thermostable polypeptides having DNA polymerase activity. In one aspect, the invention is directed to a *Thermus flavus* DNA polymerase protein

substantially free of other *Thermus flavus* proteins. Exemplary proteins include a DNA polymerase protein having the amino acid sequence of SEQ ID NO: 2. Similarly, the invention is directed to a thermostable polypeptide having DNA polymerase activity and consisting essentially of the amino acid sequence of SEQ ID NO: 2.

In addition to the cloning and expression of the Tfl DNA pol I holoenzyme, a vector allowing for the expression of a truncated DNA polymerase was generated. As explained in Example 8 and FIGURE 1B, a vector lacking the 5´ one-third of the *T. flavus* DNA polymerase I gene was constructed. Specifically, the ATG start codon of lacZ was brought in frame with the DNA encoding amino acids 239 to 834 of the Tfl DNA pol I holoenzyme using site-directed mutagenesis, and the resulting plasmid, designated p21EHcM1.1, was expressed in *E. coli* DH5αF´. The insert of plasmid p21EHcM1.1 includes a DNA sequence that corresponds with SEQ ID NO: 3 in the Sequence Listing, and encodes a polypeptide predicted to have the amino acid sequence depicted in SEQ ID NO: 4. The expressed polypeptide product was designated *Thermus flavus* DNA polymerase I exonuclease-free fragment, or "Tfl exo⁻ fragment." An aspect of the invention is directed to a purified DNA comprising a portion of the insert of plasmid p21EHcM1.1, the plasmid having ATCC Accession No. 69632, the portion encoding a thermostable polypeptide having DNA polymerase activity. This DNA may be operatively linked to additional DNAs, such as known promoter DNAs and/or expression vectors. A preferred DNA is plasmid p21EHcM1.1 itself.

As detailed in Example 8, the purification protocol for the Tfl exo⁻ fragment expressed in *E. coli* [p20EHcM1-1] included PEI-precipitation, gel filtration, Procion-Red Sepharose chromatography and immunoaffinity chromatography. The yield using this preparation protocol was approximately

300,000 units of enzyme from 50 g of cells, and the preparation had a DNA polymerase specific activity of 600,000 U/mg protein.

The N-terminal amino acid sequence of the Tfl exo⁻ fragment was determined (Example 8), and interestingly, the purified protein lacked 37 N-terminal amino acids predicted from the DNA encoding the exo⁻ fragment. The deduced amino acid sequence of the purified Tfl exo⁻ fragment -- based on this amino acid sequence data and the complete DNA sequence -- is depicted in SEQ ID NO: 5, and corresponds with amino acid 275 to 834 of FIGURE 2. A minor sequence lacking 38 N-terminal amino acids was also detected.

The foregoing demonstrates that another aspect of the invention is directed to a purified DNA encoding a thermostable polypeptide having DNA polymerase activity, the DNA comprising a portion of SEQ ID NO: 3. For example, the invention is directed to a purified DNA comprising nucleotides 112 to 1791 of SEQ ID NO: 3. This DNA also may be operatively linked to other DNAs, such as to nucleotides 1 to 111 of SEQ ID NO: 3, and/or to expression vectors known in the art. In a related aspect, the invention is directed to a vector comprising nucleotides 112 to 1791 of SEQ ID NO: 3, the nucleotides encoding a polypeptide having thermostable DNA polymerase activity. Similarly, the invention is directed to a vector having at least one insert consisting essentially of nucleotides 112 to 1791 of SEQ ID NO: 3, the nucleotides encoding a thermostable polypeptide having DNA polymerase activity.

The recombinant expression and purification of biologically active Tfl exo⁻ fragment demonstrates additional aspects of the present invention. For example the present invention is directed to a purified fragment of *Thermus flavus* DNA polymerase I protein, the fragment having DNA polymerase activity. Exemplary fragments include a fragment having an amino acid sequence comprising amino acids 2 to 560 of or 1 to 560 of SEQ. ID NO: 5, and a fragment encoded by the insert of plasmid

p21EHcM1.1, having ATCC Accession no. 69632. Also, the invention is directed to a polypeptide having DNA polymerase activity and consisting essentially of the amino acid sequence of SEQ ID NO: 5.

The foregoing description of methods and recombinant cells demonstrates that the present invention is directed to more than DNA's and polypetides. Another important aspect of the invention is directed to a host cell transformed with a DNA, vector, or plasmid of the present invention, including those specifically mentioned above. Preferably, the host cell transformed with a DNA is capable of expressing a thermostable polypeptide encoded by the DNA, the polypeptide having DNA polymerase activity. By host cell is meant both prokaryotic host cells, including *E. coli* cells, and eukaryotic host cells.

In addition to being directed to DNAs, transformed cells, and polypeptides, the present invention is directed to various methods for using DNAs and polypeptides. For example, the invention is directed to a method for purifying a thermostable polypeptide having DNA polymerase activity comprising the steps of: transforming a host cell with a DNA to create a transformed host cell, the DNA selected from the DNA's of the present invention; cultivating the transformed host cell under conditions to promote expression of a thermostable polypeptide encoded by the DNA, the polypeptide having DNA polymerase activity; and purifying the thermostable polypeptide with a monoclonal antibody that is cross-reactive with the thermostable polypeptide. In one preferred method, the cross-reactive monoclonal antibody has specificity for a *Thermus aquaticus* DNA polymerase and/or for a *Thermus flavus* DNA polymerase.

In another preferred method, commercially available chromatography columns are used to purify the expressed polypeptide.

The purification protocols for recombinant Tfl DNA polymerase I and Tfl exo⁻ fragment demonstrate that another aspect of the invention relates to methods of purifying a thermostable polypeptide having DNA polymerase

activity. One such method includes the steps of expressing the thermostable polypeptide in a host cell, the polypeptide having an amino acid sequence encoded by a DNA of the present invention; lysing the cell to create a suspension containing the thermostable polypeptide and host cell proteins and cell debris; contacting a soluble portion of the suspension with an antibody that is immunologically cross-reactive with the thermostable polypeptide under conditions wherein the antibody binds to the thermostable polypeptide to form an antibody-polypeptide complex; isolating the antibody-polypeptide complex; and separating the thermostable polypeptide from the isolated antibody-polypeptide complex to provide a purified thermostable polypeptide. Preferably, such a method further includes the steps of heating the suspension to denature the host cell proteins; and centrifuging the suspension to remove the cell debris and denatured host cell proteins. In more preferred methods, the immunologically cross-reactive antibody is a monoclonal antibody, such as a monoclonal antibody that is specific for *Thermus aquaticus* DNA polymerase I and/or *Thermus flavus* DNA polymerase I. This preferred method is exemplified herein using a monoclonal antibody purified from a hybridoma designated hybridoma 7B12. This monoclonal antibody is commercially available from Molecular Biology Resources, Inc., Milwaukee, Wisconsin, as Cat. No. 4100-01.

The invention is also directed toward a method of using a DNA encoding a DNA polymerase enzyme to generate active fragments of the DNA polymerase enzyme, comprising the steps of: deleting a portion of the DNA to create a modified DNA, expressing the modified DNA to produce a DNA polymerase enzyme fragment, purifying the DNA polymerase enzyme fragment, assaying the DNA polymerase enzyme fragment for DNA polymerase activity, and selecting a DNA polymerase enzyme fragment having DNA polymerase activity, wherein the DNA is selected from DNAs of the present invention.

As detailed in Example 9 and summarized in Table 3A, a number of experiments were conducted to characterize the exonuclease activities of *T. flavus* DNA pol I holoenzyme and exo⁻ fragment. For both the holoenzyme and the exo⁻ preparation, each exonuclease and endonuclease activity assayed was either very low or undetectable.

As detailed in Example 10, a number of additional assays were performed to better characterize the recombinant Tfl DNA pol I proteins that had been purified and to compare these proteins to other known thermostable DNA polymerases. For example, the DNA polymerase activity of the Tfl holoenzyme and the exo⁻ fragment was analyzed at different pH values, and at different $MgCl_2$ and $MnCl_2$ concentrations. FIGURES 5 (pH optima); 6A ($MgCl_2$ optima); 6B ($MnCl_2$ optima) and 7A (temperature optima) summarize the results of some of these assays. The optimal range and the peak values (in parentheses) are summarized in Table 1A.

TABLE 1A

	Holoenzyme	Exo⁻ Fragment
pH	9.5 - 10.5 (10)	7.5 - 10 (8.5)
$MgCl_2$ [mM]	>50	1.3 - 13 (5)
$MnCl_2$ [mM]	0.8 - 4 (2)	2.1 - 11 (4)

To assay thermostability the enzymes were incubated for 30 min. at different temperatures to define the temperature optimum. The highest activity (100%) was found at 80°C for the holoenzyme (14% remaining after 30 min. at 90°C), and 70 to 75°C (8% remaining after 30 min. at 90°C) for the exo⁻ fragment.

The Tfl holoenzyme preparation enzyme was more than 95% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) on 12.5% gel (FIGURE 9). The apparent molecular weight was 80 kD, which is lower than the calculated molecular weight of approximately 94 kD based on the DNA sequence. The Tfl holoenzyme preparation was found to be free of detectable double-stranded nucleases and of 5′ → 3′ exonuclease and endonuclease activities. Low levels of single-stranded nucleases and of 3′ → 5′ exonuclease activity were found. The isoelectric point was determined to be 6.43.

The purified Tfl exo⁻ fragment was found to possess low 3′ → 5′ and 5′ → 3′ exonuclease activities. The preparation was more than 95% pure as judged by SDS-PAGE (FIGURE 9). The apparent molecular weight of 68 kD as judged by SDS-PAGE compares well with the calculated molecular weight of approximately 63 kD. The Tfl exo⁻ preparation was found to be free of detectable double- and single-stranded nucleases and endonuclease activities. The isoelectric point was determined to be 5.94.

The performances of the Tfl holoenzyme and the exo⁻ fragment were tested in DNA sequencing, PCR and TCL (Examples 11, 12 and 13). Both enzymes were found to be useful in sequencing reactions utilizing labeled primer in conjunction with single-stranded and double-stranded DNA templates, and in a cycle sequencing reaction with a single-stranded template. The enzymes were also useful in sequencing reactions utilizing internal labeling with, for example, [α^{35}S]-dATP. In all the reactions tested the Tfl exo⁻ fragment provided DNA sequence information of more than 150 nucleotides, as did recombinant Tfl DNA pol I holoenzyme.

The Tfl DNA pol I holoenzyme and the exo⁻ fragment were tested in PCR reactions. The recombinant holoenzyme gave similar results to the native enzyme. The Tfl exo⁻ fragment retained 50% of its activity after 16 cycles. The holoenzyme retained 50% of its activity for 20 cycles. The specific amplified products were analyzed at the same time. After 20 cycles, an amplification product was visible on agarose gels. The amount of product increased between 25-50 cycles, but decreased after 100 cycles.

The native *T. flavus* enzyme provided with the ZEPTO™ Labeling kit (CHIMERx, Madison, WI) was replaced by the recombinant holoenzyme or by the (recombinant) exo⁻ fragment. The efficiency of the labeling of plasmid pUC19 was determined on agarose gels and the efficiency of incorporation was determined in dot blot analysis. A dilution of $1:10^6$ of labeled probes generated with the holoenzyme was detectable ($1: 10^5$ for probes generated by the exo⁻ fragment). Both results indicated that the enzymes have the required activity needed for labeling pUC19 DNA in TCL.

A protocol is also provided for demonstrating that the present invention is also directed to TCL in which the recombinant Tfl DNA pol I holoenzyme is employed without exogenous primers for enzymatic extension. In this method, referred to as Universal Thermal Cycle Labeling (UTCL), DNA of unknown sequence is combined intact with rTfl DNA Pol I holoenzyme, deoxyribonucleotide triphosphates, and the appropriate buffer. The holoenzyme is then combined with intact template and subjected to repeated cycles of denaturation annealing and extension. Alpha ^{32}P-dATP, ^{32}P-dTTP, ^{32}P-dGTP, ^{32}P-dCTP, biotin-dUTP, fluorescein-dUTP, or digoxigenin-dUTP is also included in the extension step for subsequent detection purposes.

The foregoing results demonstrate further aspects of the invention. For example, the invention is further directed to a method for labeling DNA, comprising the steps of: digesting an aliquot of template DNA with a restriction endonuclease reagent wherein the digestion generates sequence-specific DNA fragments; mixing an aliquot of undigested template DNA with the sequence-specific DNA fragments; denaturing the mixture of template DNA and sequence-specific DNA fragments thereby generating denatured template DNA and oligonucleotide primers; annealing the primers to the denatured undigested template DNA to form a DNA-primer complex; and performing an extension reaction from the primers in the DNA-primer

complex using Tfl exo⁻ fragment in the presence of one or more nucleotide triphosphates, wherein at least one nucleotide triphosphate has a label.

Further, the invention is directed to a method for thermal cycle labeling DNA comprising the steps of: digesting an aliquot of template DNA with a restriction endonuclease reagent wherein the digestion generates sequence-specific DNA fragments; mixing an aliquot of undigested template DNA with the sequence-specific DNA fragments; denaturing the mixture of template DNA and the DNA fragments thereby generating denatured template DNA and oligonucleotide primers; annealing the primers to the denatured undigested template DNA to form a DNA-primer complex; performing an extension reaction from the primers in the DNA-primer complex using Tfl DNA pol I exo⁻ fragment in the presence of one or more nucleotide triphosphates wherein at least one nucleotide triphosphate has a label; heat-denaturing the labeled extension products; reannealing the excess primers with the template DNA and with the extension products; and performing at least one additional extension reaction from the DNA-primer complex using a Tfl DNA pol I exo⁻ fragment.

The present invention is further directed to kits for labeling DNA. A kit of the present invention includes, in association: a labeling buffer; a concentrated mixture of 1 or more nucleotide triphosphates; Tfl DNA pol I exo⁻ fragment; and a control DNA, the control DNA being useful for monitoring the efficiency of labeling. Additionally, the kit may include a restriction endonuclease reagent and a restriction endonuclease buffer.

In another aspect, a kit of the present invention for labeling DNA comprises, in association: a Tfl DNA pol I exo⁻ fragment; and a Tfl DNA pol I exo⁻ fragment buffer. Preferably, such a kit further comprises a concentrated mixture of 1 or more nucleotide triphosphates and a control DNA, the control DNA being useful for monitoring the efficiency of labeling.

The following examples are intended to describe various aspects of the invention in greater detail. More particularly, in Example 1, the

purification and amino acid sequencing of native *Thermus flavus* DNA polymerase I is described. In Example 2, the construction and amplification of a *Thermus flavus* genomic DNA library is described. In Example 3, the cloning and sequencing of a *Thermus flavus* DNA polymerase I gene fragment is described. Example 4 details the preparation of gene-specific probes and screening of the *Thermus flavus* genomic library for clones containing the *T. flavus* DNA pol I gene. Example 5 details the sequencing of the *T.flavus* DNA polymerase I gene. In Example 6, the construction and expression of a full-length *T. flavus* DNA pol I clone and purification of full-length recombinant *T. flavus* DNA pol I protein are described. In Example 7, the construction and expression of a high-yield, full-length *T. flavus* DNA pol I clone and purification of full-length recombinant *T. flavus* DNA pol I is described. Example 8 details the cloning and expression of the exo$^-$ fragment of *T. flavus* DNA polymerase I. In Example 9, the characterization of recombinant *T. flavus* DNA polymerase I exonuclease activities is detailed. In Example 10, studies are described comparing the recombinant *T. flavus* and *T. aquaticus* DNA polymerases. In Example 11, DNA sequencing with recombinant *T. flavus* DNA polymerases is detailed. Example 12 demonstrates the utility of recombinant Tfl holoenzyme and the exo$^-$ fragment in polymerase chain reaction procedures. Example 13 demonstrates the utility of recombinant Tfl DNA pol I holoenzyme and the Tfl exo$^-$ fragment for use in thermal cycle labeling procedures. Example 14 analyzes the utility of *T. flavus* DNA pol I holoenzyme and exo$^-$ fragment for reverse transcription applications. Example 15 demonstrates the increased processivity of Tfl exo$^-$ fragment as compared to native or recombinant Tfl DNA pol I holoenzyme or Taq holoenzyme. Finally, Example 16 details a large "production scale" purification of recombinant Tfl holoenzyme and exo$^-$ fragment.

EXAMPLE 1

Purification and Amino Acid Sequencing of Native Tfl DNA Pol I

Native *T. flavus* DNA polymerase I was isolated from *T. flavus* cells and used to generate amino acid sequence information as described below.

Thermus flavus obtained from the American Type Culture Collection (ATCC 33923, Catalogue of Bacteria and Bacteriophages, 18th Edition, 1992) was cultured as follows: one ampule of *Thermus flavus* ATCC 33923 was used to inoculate 100 ml culture medium (0.1 g nitrilotriacetic acid, 3 g NZ Amine A, 3 g yeast extract, 5 g succinic acid [free acid], 0.001 g riboflavin, 0.522 g K_2HPO_4, 0.480 g $MgSO_4$, 0.020 g NaCl, 2 ml Trace Metal Solution (0.5 ml H_2SO_4, 2.2 g $MnSO_4$, 0.5 g $ZnSO_4$, 0.5 g H_3BO_3, 0.016 g $CuSO_4$, 0.025 g Na_2MoO_4, 0.046 g cobalt nitrate) per liter, adjusted to pH 8.0 with NaOH) and the culture was incubated overnight at 70°C with shaking. In the morning 10 ml of the overnight culture was used to inoculate 1000 ml of medium. This culture was grown for about 8 hours at 70°C and then used as an inoculum for 170 liters of medium in a New Brunswick 250 liter fermentor equipped with a ML 4100 controller. The settings for a typical fermentation were 3 pounds back pressure, 60 liters/min. (lpm) aeration, 100 rpm agitation, at 70°C. The fermentation was terminated when the cells reached a density of 2 - 3 O.D., as measured at 600nm. The cells were cooled down to room temperature and harvested by centrifugation at 17,000 rpm in a CEPA type 61 continuous flow centrifuge with a flow rate of 2 lpm. The cell paste was stored at -70°C until used.

T. flavus cells (500-1500g) were thawed in 3 volumes of lysis buffer (20mM Tris-HCl, pH 8.0, 0.5mM ethylenediaminetetraacetate (EDTA), 7 mM β-mercaptoethanol (βME), 10 mM $MgCl_2$) and homogenized. Phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, was added to a final concentration of 0.3 mM. The suspension was then treated with 0.2 g/l

of lysozyme (predissolved in lysis buffer) at 4°C for 1 hr. Cells were homogenized twice at 9000 psi in a Manton Gaulin homogenizer, with the suspension chilled to approximately 10°C between passes. New PMSF was added to 0.2 g/l before, between and after passes. NaCl and polyethyleneimine (PEI) (10% w/v, pH 7.0) were added to the crude, homogenized lysate to a final concentration of 0.5 M and to 0.2%, respectively. The sample was mixed well and centrifuged at 13,500 x g for 1 hour.

The supernatant from the centrifuged lysate was desalted by diluting with 10 liters of DE52 column buffer (20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 7 mM βME) and concentrated to approximately 4 liters using an Amicon S10Y30 Spiral Ultrafiltration cartridge. The dilution/concentration step was repeated two times, with a final concentrated volume of about 4 liters.

The desalted sample was batch contacted with 400 g of equilibrated Whatman DE52 ion exchange resin (Maidstone, England). The suspension was collected on a sintered glass funnel and washed 3 times with 1 volume of DE52 column buffer. The resin was then resuspended in a minimal volume of buffer and poured into a column (4.5 x 50 cm), packed and washed with an additional volume of buffer. The column was eluted with a 0-0.5 M NaCl linear gradient (total gradient volume: 2000 ml). Twenty-five ml fractions were collected at a rate of about 5 ml/min. Peak fractions (fractions containing DNA polymerase activity) were determined by a modified DNA polymerase assay described by Kaledin et al., *Biokhimiya* *45*:644-651 (1980), pooled and dialyzed in approximately twenty-five volumes of Affi-Gel Blue (AGB) column buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10 mM βME, 10mM $MgCl_2$, 0.02% Brij 35).

The dialyzed DE52 peak fractions were applied to an AGB column (4.4 x 40 cm, 600 ml packed volume, MBR Blue, Molecular Biology Resources, Milwaukee, WI), which was washed with 2 column volumes of

AGB column buffer, and eluted with a 0-1.2 M NaCl linear gradient (total gradient volume: 2000 ml). Twenty-five ml fractions were collected at a rate of 1-5 ml/min. The peak fractions were dialyzed as above in AGB buffer.

The dialyzed AGB peak fractions were applied to a heparin agarose column (4.4 x 16.5 cm, 250 ml packed volume (Affigel Heparin, Bio-Rad, Hercules, CA; or Heparin Agarose, Molecular Chimerics, Madison, WI)), which was washed with approximately 2 column volumes (until effluent is no longer colored, and column resin is white in appearance), and eluted with a 0.1-1.0 M NaCl linear gradient (total gradient volume: 1500 ml). Twenty-five ml fractions were collected at a rate of 1-5 ml/min. The peak fractions were dialyzed in HP Q Sepharose Column Buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 7 mM βME, 0.1% Brij 35).

The dialyzed heparin agarose peak fractions were filtered through a 0.2 μm filter and applied at 4 ml/min. to the HP Q Sepharose column (Pharmacia, Uppsala, Sweden) on FPLC. The column was washed with several column volumes of buffer, and eluted with a 0-0.25 M NaCl linear gradient. Ten ml fractions were collected at 4 ml/minute. The peak fractions were dialyzed in HP S Column Buffer (20 mM Na-Citrate, pH 6.0, 1 mM EDTA, 7 mM βME, 0.1% Brij 35) or diluted in the same buffer, depending on the volume of the fraction pool.

The dialyzed (or diluted) HP Q peak fractions were filtered through a 0.2 μm filter and the HP S column (Pharmacia) was run as above, washing with HP S Column buffer and eluting with a 0-0.25 M NaCl gradient. Peak fractions were pooled and dialyzed against 4 liters of Final Storage Buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5mM DTT, 50% glycerol). The final product was diluted to a concentration of 5000 U/ml in the above buffer including 0.5 % Tween 20 (Sigma Chemical Co., St. Louis, MO) and 0.5 % Nonidet P40 (Fluka Biochemika, Buchs, Switzerland) as stabilizers and stored at -20°C. A typical preparation from 1200 g of cells

yields approx. 2,000,000 units (1,700 units/g) or about 40 mg of DNA polymerase.

To quantify DNA polymerase activity, a DNA polymerase activity assay was performed using a modification of a protocol described by Kaledin et al., *Biokhimiya 45*:644-651 (1980). Reactions were performed in a 50 μl reaction mixture of 25 mM Tris-HCl, pH 9.5 at 23°C; 50mM KCl; 10 mM $MgCl_2$; 1 mM DTT; 0.2 mM each dCTP, dGTP, dTTP, pH 7.0; 0.2 mM [α^{32}P]dATP, pH 7.0, 10 μCi/ml; 50 μg BSA; 15 μg activated DNA (Baril et al. *Nucleic Acids Res. 8*:2641-2653 (1977)); and 5 μl of diluted enzyme. For control purposes enzymes (in general AmpliTaq DNA polymerase (Perkin Elmer, Cat. No. N801-0060), or Taq DNA polymerase purified according to a procedure described by Kaledin et al., *Biokhimiya 45*:644-651 (1980)) with known activities are diluted to 20, 40 and 80 units/ml. Two reactions were run without enzyme as negative controls for background subtraction.

A 45 μl reaction mixture, less enzyme, was prepared and the reaction was started by the addition of 5 μl of enzyme. After 10 min. of incubation at 70°C, 40 μl was removed and added to 50 μl of yeast RNA co-precipitant (10 mg/ml in 0.1 M sodium acetate, pH 5.0). One ml of 10% trichloracetic acid (TCA) was added and the samples were placed on ice for at least 10 minutes. The mixture was filtered on a glass fiber filter disc and washed first with 5% TCA/ 2% sodium pyrophosphate, and then with 95% ethanol. The dried filter disc was counted in 5 ml of scintillation fluid.

One unit of activity is defined as the amount of enzyme required to incorporate 10 nmol of total nucleotide into acid insoluble form in 30 min. at 70°C in this assay, the standard activity assay.

To estimate protein concentration, an aliquot of a native *T. flavus* DNA polymerase preparation (1100 U/ml) was separated on a 5 - 25% SDS-polyacrylamide gel, using the Bio-Rad protocols (Hercules, CA) and the

Bio-Rad Mini-Protean II electrophoresis unit. The concentration was estimated at 33 μg/ml when compared to co-electrophoresed protein standards.

To obtain amino acid sequence information from native *T. flavus* DNA polymerase, about 53 μg of native polymerase were separated on a preparative 7.5% SDS-polyacrylamide gel, blotted onto PVDF membrane and stained with amido black as described by Matsudaira, *J. Biol. Chem. 262*: 10035-10038 (1987). The major band at approximately 83 kD was excised and sequenced using an Applied Biosystems (Foster City, CA) 477A Protein Sequencer. No N-terminal sequence was obtained under these conditions.

Due to the apparent block at the N-terminus of the native *T. flavus* DNA polymerase I (holoenzyme), another approach was employed to obtain a partial amino acid sequence. Native *T. flavus* DNA polymerase I was digested with trypsin, and the resulting peptides were separated using reverse phase high-performance liquid chromatography (HPLC). The N-terminal amino acid sequences of four of these peptides (peptides 1-4) were determined. The amino acid sequence of one of the peptides, peptide 1, is LHTRFNQTATATGRLSSSDPNLQNIPVR. This sequence has been determined to map at positions 562 to 589 in the deduced amino acid sequence of the Tfl DNA pol I holoenzyme described herein (FIGURE 2)). As explained in Example 3, knowledge of this amino acid sequence information was used to isolate the *T. flavus* DNA polymerase I gene.

EXAMPLE 2

Construction and Amplification of a *Thermus flavus* genomic DNA library

A *Thermus flavus* genomic library was constructed in phage λ Dash II and amplified in the following manner.

Genomic DNA from the *Thermus flavus*, cultured overnight as described above, was isolated according to the procedure described by Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publishing

Associates and John Wiley & Sons, New York (1990). In general, yields of genomic DNA between 100 and 900 μg were obtained from the cell pellet of about 1.5 ml of culture.

Twenty-five micrograms of *Thermus flavus* genomic DNA were partially digested with 0.3 units of Sau3A1 in a total reaction volume of 50 μl. At 0, 5, 10, 15, and 30 min., 10μl samples were removed and the enzyme was inactivated at 65°C for 15 min. An aliquot from each time point was analyzed on a 1.2% agarose/TBE gel. The 10 min. reaction time produced fragments having the desired size distribution (3 kb to 20 kb).

Approximately 2.5 pmoles of 5´-ends of Sau3A1-digested *T. flavus* DNA were treated with calf intestinal alkaline phosphatase (CIP) using standard techniques (Ausubel *et al.*, Current Protocols in Molecular Biology (1990)). One half of the CIP-digested sample was electrophoresed on a 0.7% agarose gel and checked for amount and integrity. The Sau3A1- digested, CIP-treated *T. flavus* DNA was extracted with phenol/chloroform and chloroform, ethanol precipitated, pelleted, and washed in 70% ethanol. The pellet was stored at -20°C. This DNA is referred to as "CIP TFL DNA."

The *T. flavus* library was constructed as described in the manufacturer's instructions using the phage λ DASH II / BamHI Cloning Kit (Stratagene, LaJolla, CA) and the CIP Tfl DNA. The pME/BamHI test insert (0.3 μg) was run in parallel as a control. The ligation mixture was incubated over night at 4°C.

The *T. flavus* DNA ligated to λ DASH II arms was packaged *in vitro* using the Gigapack II Gold Packaging Extract from Stratagene, according to the manufacturer's instructions. Control DNA provided by the manufacturer was also packaged.

Following the protocol provided by Stratagene with the λ DASH II / BamHI Vector Kit, host bacteria were prepared: *Escherichia coli* VCS 257 (Stratagene) for wild type phage; *E. coli* SRB and SRB(P2)

(Stratagene) for the *T. flavus* library and the control. VCS 257 was grown in NZY+ maltose medium; SRB and SRB(P2) were grown in NZY+ maltose medium with 50 μg/ml kanamycin at 37°C for 6 hours. After centrifugation of the cells at 2800 x g for 10 min., the cells were resuspended in sterile 10 mM $MgSO_4$ to give an A_{600} (optical density at 600nM) of 0.5.

Two 1:10 serial dilutions were prepared from the control phage and the CIP Tfl DNA library. Ten microliters of undiluted, 1:10, and 1:100 dilutions of phage were added to 200 μl of SRB cells. The cells were incubated with light shaking at 37°C for 15 minutes and after the addition of top agar, the mixture was poured onto LB/M/M plates. The plates were incubated overnight at 37°C.

The *T. flavus* library was amplified using techniques described by Ausubel *et al.*, Current Protocols in Molecular Biology (1990), and the primary and amplified libraries were titered on SRB cells and the titers are shown in Table 1B. The amplified library was stored at 4°C.

TABLE 1B
Titer (plaque forming units/ml)

Construct	Primary Library	Amplified Library
CIP TFL DNA	4.4×10^5	9.6×10^7
pME/BamHI	3×10^6	1.5×10^9
λ Control DNA	1.1×10^9	Not determined

EXAMPLE 3

Cloning and Sequencing a Tfl DNA Pol I Gene Fragment

The amino acid sequence information derived from four Tfl DNA pol I peptides (Example 1) was used to design the synthesis of two primers for the amplification of a *T. flavus* DNA polymerase gene fragment:

primer FTFL2 (primer "2"; 21mer) (SEQ ID NO: 8) and primer RTFL4 (primer "4"; 25mer) (SEQ ID NO: 9) (synthesized by Synthetic Genetics, San Diego, CA). The sequence of the two primers was also compared to the *T. aquaticus* DNA polymerase sequence (Lawyer et al., *J. Biol. Chem 264*: 6427-6437 (1989)); the primer nucleotide sequences, with cross-references to the Sequence Listing and Sequence ID Nos. are shown in Table 2. Primer FTFL2 was chosen because the amino acid sequence obtained from peptide 1 (Example 1) was identical to a sequence in the Taq DNA polymerase I protein. Primer FTFL2 corresponds to nucleotides 1719-1740 of the *T. aquaticus* DNA polymerase coding sequence, top strand (i.e., to a portion of the sequence that encodes a portion of the Taq DNA pol I protein that is homologous to Peptide 1). Primer RTFL4 hybridizes to the 3´-end of the Taq DNA pol I gene at position 2476 - 2500 and has sequence identical to the bottom strand (Lawyer *et al.*, *J. Biol. Chem 264*: 6427-6437 (1989)).

A typical amplification reaction (100 μl) contained 0.2 mM deoxynucleotide triphosphates (dNTPs), 1 x Taq Polymerase Reaction Buffer (10 x buffer is 100 mM Tris-HCl, pH 8.4, 500 mM KCl, 15 mM $MgCl_2$), 0.5 μM of each primer FTFL2 and RTFL4 (primer set 2-4), 50 μl mineral oil and 15 ng *T. flavus* genomic DNA. After the initial denaturation step (Step 1), 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer No. N801-0060, Foster City, CA) were added. Negative control reactions containing either no enzyme or no template were performed. The amplification program was carried out in a thermocycler as follows: Step 1: 95°C for 5 min.; Step 2: hold at 72°C (for the time required to add the enzyme); Step 3: 55°C for 45 sec.; Step 4: 72°C for 5 min.; Step 5: 95°C for 15 sec.; Step 6: repeat Steps 3-5 34 times; Step 7: 55°C for 45 sec.; Step 8: 72°C for 20 min.; Step 9: hold at 4°C until processing the product. Under these conditions primer set 2-4 gave a single amplification product from *T. flavus* genomic DNA. The observed mobility of the amplification product ("the 2-4 fragment") in 1% and

1.2% agarose gels was in agreement with the 782 bp predicted from the *T. aquaticus* coding sequence.

The 2-4 fragment was cloned, sequenced, and compared to a previously published DNA sequence for a purported *T.flavus* DNA polymerase I as follows.

First, to improve cloning efficiency, the 2-4 fragment was fractionated, blunt ended, and phosphorylated as follows. Approximately 20 μl of the 2-4 fragment was loaded onto a Sephacryl S-500 (400 μl in a spin filter, preswollen, pre-equilibrated and stored in 100 mM Tris-HCl, pH 8.0, 1 mM EDTA) column and centrifuged at 2,000 x g for 5 min. to trap the unused primers from the PCR reaction. DNA that passed through the column was ethanol-precipitated and resuspended in double-distilled water (ddH_2O). The 2-4 fragment was blunt-ended using mung bean nuclease (MBN) (Molecular Biology Resources, Inc., Cat. No. 1190-01.), and phosphorylated with T4 polynucleotide kinase (Molecular Biology Resources, Inc., Cat. No. 1260-01) prior to ligation to a vector by procedures well known in the art.

M13mp18 RF DNA (Life Technologies, Grand Island, NY) was restriction-digested with Hinc II and Ecl 136 II to create blunt ends for ligation to the above 2-4 fragment. The vector ends were dephosphorylated with CIP to reduce the probability of self-ligation. The digested and dephosphorylated M13mp18 vector and the 2-4 fragment (fractionated/MBN/kinased) were ligated for 2 hr. at room temperature using procedures that are well known in the art.

Using standard techniques, 5 μl of the ligation reaction were added to 50 μl of DH5αF′ *E. coli* (Life Technologies, Grand Island NY) cells, which had first been made competent, and the cells were transformed (see Ausubel et al., Current Protocols in Molecular Biology (1990)). Different numbers of cells were spread on 2XTY plates and grown until plaques appeared. Several plaques were picked, and DNA was prepared using the Minute Miniprep ssDNA Purification Kit (CHIMERx, Madison WI). It

was determined that plaques designated M13-TFL 4.21 and 4.22 contained the 2-4 fragment in opposite orientations.

The DNAs M13-TFL 4.21 and 4.22 were sequenced by Sanger's dideoxy method (Sanger et al., *Proc. Natl. Acad. Sci. 74*:5463 (1977)) using the SEQUAL™ Sequencing Kit from CHIMERx. The forward sequencing primers (FSP, Table 2, SEQ ID NO: 6) used in sequencing M13-TFL 4.21 and M13-TFL 4.22 were end-labeled using [γ^{32}P]-ATP and T4 polynucleotide kinase. The extension/termination reactions were performed according to the protocol provided with the SEQUAL™ Sequencing Kit (CHIMERx). One microliter of each extension/termination reaction was loaded onto a 6% sequencing gel, which was electrophoresed at 3000 volts for 3 hours. The bands were detected by autoradiography and the sequence was determined.

When the nucleotide sequence from both ends of the 2-4 DNA was aligned, 771 bp of the approximately 780 bp DNA could be determined. This 2-4 DNA sequence was compared with the purported *T. flavus* DNA polymerase sequence reported by Akhmetzjanov and Vakhitov, *Nucleic Acids Res.* 20:5839 (1992), which theoretically should have been amplified by this primer set. Eighty-four percent maximum matching, as calculated by the MacDNAsis software program (Hitachi Software Engineering America, San Bruno, CA), was found. This degree of homology, compared with a reported DNA polymerase gene, suggested that the 2-4 DNA was indeed part of the *T. flavus* (ATCC 33923) gene and could serve as a useful probe for screening the *T. flavus* genomic library. The homology of 84% also suggested that either (1) the purported *T.flavus* strain studied by Akhmetzjanov and Vakhitov and the *T. flavus* strain (ATCC 33923) do not have identical DNA polymerase I genes; or (2) the two strains have more than one gene or gene-like sequences having homology to DNA polymerase I genes.

EXAMPLE 4

Preparation of Gene-Specific Probes and Screening of the *Thermus flavus* Genomic Library for Clones Containing the *T. flavus* DNA Pol I Gene

The 2-4 fragment described in Example 3 was used to isolate the *Thermus flavus* DNA pol I gene from the *T. flavus* genomic library (Example 2). Using M13-TFL 4.21 as template and primers FTFL2 and RTFL4, the 2-4 fragment was amplified by PCR as described above to obtain larger quantities of the fragment for use in preparing probes to screen the *T. flavus* genomic library. The amplified 2-4 fragment, migrating at about 780 bp, was cut out of preparative 0.7% agarose gel, eluted, phenol-chloroform extracted and ethanol-precipitated. Approximately 1 μg of the 2-4 fragment was digested with *CviJI*® (CHIMERx) to generate sequence specific primers for labeling. A variety of thermal cycle labeling (TCL) probes were prepared with the 2-4 intact fragment (i.e., Biotin-11-dUTP, fluorescein and [α^{33}P]dCTP probes) in the manner described below. Each set of duplicate plaque lifts or targets was screened using two different types of labeled probes. Digestion with CviJI*, as well as this method of labeling, is described in a co-owned, copending U.S. Patent Application Ser. No. 08/217,459, filed March 24, 1994, entitled "Methods and Materials for Restriction Endonuclease Applications," incorporated herein by reference in its entirety. The PCT counterpart of this application, filed March 24, 1994, is PCT App. No. US94/03246.

The 2-4 intact fragment was labeled with Biotin-11-dUTP as described in the manual for the ZEPTO™ Labeling Kit (CHIMERx). To determine the relative efficiency of the amplification reaction, 5μl of the amplified 2-4 TCL probe was electrophoresed on a 0.7% agarose gel along side a 1 kb molecular size ladder. The amplified probe was evident as a smear from 0.1-5 kb, which is an indication of a successful TCL reaction.

To determine the efficiency of incorporation of the biotin-11-dUTP, a dot blot assay was performed as follows: A serial dilution of the probe from 1:10 to $1:10^8$ was made in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 1μl of each dilution was spotted on a Hybond-N membrane (Amersham, Arlington Heights, IL), UV-cross-linked for 3 min., followed by colorimetric detection of the incorporated biotin-11-dUTP using streptavidin-alkaline phosphatase as described in the ZEPTO™ labeling manual. The probe was detected at $1:10^6$ dilution suggesting that the biotin-labeled 2-4 fragment was efficiently labeled and is highly sensitive for the screening of the Tfl genomic library.

The fluorescein labeled 2-4 fragment was prepared and analyzed as above except fluorescein-12-dUTP was used instead of biotin-11-dUTP. The fluorescein-12-dUTP incorporation was detected using alkaline phosphatase conjugated anti-fluorescein antibody (Boehringer-Mannheim, Indianapolis, IN) instead of streptavidin-alkaline phosphatase. These probes were detected at a $1:10^6$ dilution by the colorimetric assay as described in the ZEPTO™ labeling manual or by chemiluminescence. Both the biotin and flurescein non-radioactive probes were aliquoted and used throughout the entire screening process.

The preferred detection method for both the biotin-11-dUTP probes and the fluorescein-12-dUTP probes was chemiluminescence. For this method of detection the filters with hybridized probes were incubated either with streptavidin-alkaline phosphatase or alkaline phosphatase conjugated to anti-fluorescein antibody for 30 min. at room temperature. They were then rinsed three times with wash buffer (1 x phosphate buffered saline (PBS), 0.3% Tween 20 (Sigma Chemical Co., St. Louis, MO) 0.02% Na-azide) for 15 min. each and finally in assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$ and 0.02% Na-azide, pH 10) for 5 minutes. They were finally incubated in assay buffer containing CSPD™ (Tropix, Bedford, MA) a chemiluminescence substrate, for 15 min. in the dark followed by exposure to X-ray films. The

normal exposure times for the biotin-11-dUTP probes were 5-30 min. and for the fluorescein-12-dUTP probes were 2-6 hours.

The 2-4 intact fragment was labeled with [α^{33}P]dCTP as described in the ZEPTO™ labeling manual; a total of 6 x 10^7 cpm of [α^{33}P]dCTP at 1 x 10^9 cpm/μg was incorporated. For probes, 1-5 x 10^6 cpm of radio-labeled DNA was added to each plaque lift.

The sensitivity and specificity of the labeled probes was demonstrated by screening blots of digested *T. flavus* genomic DNA. Specifically, *T. flavus* genomic DNA was restricted with different restriction enzymes, such as BamHI, BglI, DraI, EcoRI, EcoRV and PacI. 250 ng/lane of restricted DNA, along with 500 ng of IL-3A viral DNA as negative control (Xia, Y., et al., *Nucleic Acids Research 15*: 6075-6090 (1987)), were electrophoresed on a 0.7% agarose gel. A Southern transfer of this gel onto Hybond-N was prepared. The denatured DNA on the Southern blots was UV-cross-linked to the filter for 3 minutes. Duplicate blots were prehybridized in 2 ml of hybridization buffer (50% deionized formamide, 7% SDS, 120 mM Na phosphate, pH 7.2, 250 mM NaCl, 1 mM EDTA and 1 mM cetyldimethylethylammonium bromide and 20μl of denatured salmon sperm DNA at 10 mg/ml) in a heat-sealed plastic bag at 52°C for 1 hour. Seven μl of either the biotin-11-dUTP 2-4 TCL probe or the fluorescein-12-dUTP 2-4 probe was added to one set of the Southern blots and 1-5 x 10^6 cpm of the [α^{33}P]dCTP 2-4 TCL probe was added to the duplicate blot. The filters were hybridized by incubation overnight at 52°C.

The filters with the radioactive probe were incubated with low stringency buffer (1 x SSC, 1% SDS) for 1 hr. at 52°C, washed with high stringency buffer (0.1 x SSC, 1% SDS) for 1 hr. at 50°C, dried, and then exposed to X-ray film for 3 hours.

The detection of non-radioactive probes was accomplished as described above. Both the biotin-11-dUTP and the [α^{33}P]dCTP 2-4 TCL probes recognized a large molecular weight band at about 20 kb in all the

lanes containing digested *T. flavus* genomic DNA, although the mobility of the bands varied somewhat in the lanes containing different digests. The probes did not bind to the control IL-3A DNA, suggesting that the probes were specific for the target and could be used to screen the *T. flavus* genomic library.

To screen the amplified *T. flavus* genomic library (Example 2), the phage library was plated on two plates each at 10^5 plaque-forming units (pfu)/100 mm 2XTY plates. Duplicate plaque lifts on Hybond N from each plate were obtained and prepared for hybridization by methods well known in the art (Sambrook, Fritsch, and Maniatis, Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Press (1989)). The DNA on the plaque lifts was UV-cross-linked to the Hybond N for 3 minutes and each plaque lift was placed in a heat-sealed plastic bag and prehybridized as described above. Seven μl of either the 2-4 biotin-11-dUTP TCL probe or the 2-4 fluorescein-12-dUTP probe were added to one set of the plaque lifts and 1-5 x 10^6 cpm of the [α^{33}P]dCTP 2-4 TCL probe were added to the duplicate filters. The filters were incubated overnight at 52°C and washed the next day with low and high stringency buffers as described above. The filters with non-radioactive probes were incubated with 10 ml of conjugation buffer (0.5% casein, 1 x PBS and 0.02% Na-azide) for 30 min. at room temperature. Hybridization conditions, washes and detection were as described above.

Approximately 25 positive plaques (hybridizing with the labeled probes) out of 10^5 pfu from the amplified CIP Tfl DNA library were detected on the duplicate plaque lifts.

Ten positive plaques were selected (λ21, λ31, λ51, λ61, λ71, λ81, λ91, λ101, λ111 and λ121) and were purified by two rounds of dilution and screening with the labeled 2-4 probes, until well-isolated, single, positive plaques were obtained.

The four stocks of phage λ21, λ51, λ71, and λ91 were grown at 5 x 10^5 pfu/2XTY plate, 5 plates per stock. The phages were eluted from

the plates by a standard protocol (Sambrook et al. (1989)). The eluant was treated with 20μg/ml DNase and 50μg/ml RNAse A for 1 hr. at 37°C and extracted with both phenol-chloroform and chloroform. The DNA was ethanol-precipitated, pelleted, rinsed with ethanol, resuspended in 1 ml of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and purified using the Lambda prep kit from CHIMERx.

The phage DNA was restriction-digested with EcoRI and BamHI and electrophoresed on a 0.7% agarose gel, transferred to Hybond N and probed with the 2-4 TCL probes. Based on agarose gel band distribution and Southern blot detection by the 2-4 probes, the four phages were grouped into two classes. Clones λ21 and λ91 belong to one class and the λ51 and λ71 belong to a second class. Clones λ21 and λ51 were chosen for further analyses.

Clone λ21 was digested with BamHI and the *T. flavus* insert was subcloned into pTZ18U (Mead et al., *Protein Engineering 1*: 67-74 (1986)). Eight of these clones were sequenced using the SEQUAL™ Sequencing kit from CHIMERx. One of these clones, designated p21BG, hybridized to the 2-4 TCL probes and yielded sequences identical to the sequence of the 2-4 fragment between the BamHI and Eco47III sites (these sites begin at positions 2084 and 2387 in Fig. 4, respectively). This sequence information confirmed that the clones contained authentic *T. flavus* DNA polymerase gene sequence, and confirmed the orientation of this gene sequence in the clones.

Based on agarose gel analysis neither λ21 nor λ51 had any internal Eco RI sites, hence λ21 and λ51 were restriction-digested with Eco RI and the insert was cloned into pTZ18U for ease in further analyses. The resulting recombinants were designated p21E10 and p51E9, respectively (FIGURE 1A). Each clone had an insert of about 14-16 kb.

EXAMPLE 5

Sequencing the *T. flavus* DNA Polymerase I Gene

Three main strategies were adopted for sequencing the Tfl DNA pol I gene.

In a first strategy, several primers were designed based on the purported *T. flavus* DNA pol I sequence published by Akhmetzjanov and Vakhitov (1992) and were synthesized by Synthetic Genetics, San Diego, CA (e.g., primers FTFL10, FTFL11, RTFL12, RTFL13, FTFL15, RTFL16, FTFL17 and RTFL18 (Table 2)). Dideoxy- sequencing of the λ21 and λ51 clones was attempted using these primers, but only primers FTFL17 and RTFL18 yielded good sequence data and only very faint bands were obtained with primers FTFL11 and RTFL13, suggesting only partial homology to this purported DNA pol I sequence.

In a second sequencing strategy, deletion vectors of p21E10 were obtained by restriction digestion of the plasmid with HincII, HindIII, SphI, KpnI and BamHI. These restriction enzymes cut once in the multiple cloning site and once or twice in the insert. The digests were diluted to allow self-ligation and transformed into *E. coli* strain DH5αF' by standard methods. The clones that ligated back to the vector were selected on ampicillin-containing plates and picked for further sequence analysis.

All of these clones had deletions of different lengths at the 3' end. The size of the insert in the HincII deletion vector (p21EHc) was approximately 4.6 - 4.7 kb; in the KpnI deletion vector (p21EK) about 7 kb; in the HindIII deletion vector (p21EHd) about 1.4 kb; in the SphI deletion vector (p21ES) about 1.6 kb; and in the BamHI deletion vector (p21EB) about 1.2 kb. The plasmids p21EHd, p21EB and p21ES were sequenced with [γ-^{32}P] end-labeled FSP by dideoxy sequencing. The sequences obtained were within the region of the 2-4 fragment, further confirming the orientation of the

insert and the presence of the 3' region of the *T. flavus* DNA polymerase I in the p21E10 parent clone.

Clone p21EHc is a deletion derivative containing the entire portion of the Tfl DNA polymerase I gene DNA present in p21E10 and about 3 kb DNA downstream from the stop codon of the Tfl DNA pol I gene, but lacking about 9.0 kb of unwanted 3' end sequence (FIGURE 1A). In addition, the DNA sequence obtained from p21E10 and p21EHc using [γ-^{32}P] end-labeled reverse sequencing primer (RSP, Table 2) suggested that p21E10 contained only about 2/3 of the DNA polymerase gene and lacked the 5' one-third of the gene. In contrast, sequence obtained from p51E9 suggested that this clone contains a 5' portion of the Tfl DNA pol I gene that overlaps the coding sequence contained in p21E10, as well as significant Tfl DNA upstream of the gene.

A primer walking sequencing strategy was employed to obtain the remainder of the sequence of the Tfl DNA pol I gene. This strategy is described as "Directed Sequencing with Progressive Oligonucleotides" in Sambrook et al., Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Press (1989). To obtain additional sequence information from the clones by primer walking, primers homologous or complimentary to the ends of previously determined sequences obtained from p21E10, from the deletion vectors, and from primers FTFL17 and RTFL18 were synthesized as described above and used in additional sequencing reactions. By repeating this process the entire length of the gene was sequentially sequenced.

Specifically, TFL primers FTFL17A, RTFL18A, TFLEF1 and TFLER1 (Table 2 and FIGURE 4) were synthesized by Synthetic Genetics for primer walking based on the sequence information obtained from primers FTFL17, RTFL18 and RSP on the p21E10 template DNA (Table 2 and FIGURE 4). Primer TFLSF1 was designed as a forward primer for walking into the 3' end of the DNA pol I gene, by utilizing sequence information from p21ES. As more information became available, additional TFL primers

RTFLA - FTFLZ (Table 2; FIGURE 4) were designed for sequencing both strands.

The DNA sequence of the *T. flavus* DNA polymerase I gene and flanking sequences are given in FIGURE 2 (SEQ. ID NO. 1), along with the deduced amino acid sequence (SEQ. ID NO. 2). The sequence of 3326 b.p. has been determined. FIGURE 2 depicts 3048 bases of this sequence, of which 2505 bases are deduced to encode a polypeptide of 834 amino acids (plus stop codon). The coding sequence was determined to be 86% homologous to the Taq polymerase gene and 83% homologous to the purported Tfl polymerase gene published by Akhmetzjanov and Vakhitov.

The deduced amino acid sequence of the *T. flavus* DNA polymerase I gene was aligned and compared to the deduced amino acid sequences of the purported Tfl DNA pol I published by Akhmetzjanov and Vakhitov (85% homology) and to the deduced amino acid sequence of the Taq pol I gene (87% homology). As shown in FIGURE 3, the amino acid alignment chosen to maximize homology reveals two single amino acid insertions in the *T. flavus* DNA pol I reported here, relative to the other two reported sequences. The single amino acid inserts are depicted by dashes (-) in the sequences for Taq pol I and for Akhmetzjanov and Vakhitov's purported Tfl DNA pol I.

TABLE 2

Primer name:	Primer Sequence	Seq. Id. No:
FSP	CGC CAG GGT TTT CCC AGT CAC GAC	6
RSP	AGC GGA TAA CAA TTT CAC ACA GGA	7
FTFL2	CTA AGT AGC TCC GAT CCC AAC	8
RTFL4	ATC ACT CCT TGG CGG AGA GCC AGT C	9
FTFL10	ATT TAG CAC ATA TGG CGA TGC TTC CC	10
FTFL11	CTT TCC AGC TCC GAC CCC AAC	11
RTFL12	CCT ACT CCT TGG CGG AGA GCC AGT C	12
RTFL13	TGG ATG TCC CTC CCC TCC TGA AAG A	13
FTFL15	CCC TTT CCC GGA AGC TTT CCC AGG TGC A	14
RTFL16	TGC ACC TGG GAA AGC TTC CGG GAA AGG G	15
FTFL17	CCT GCA GTA CCG GGA GCT CAC CAA GCT CAA	16
RTFL18	TTG AGC TTG GTG AGC TCC CGG TAC TGC AGG	17
FTFL17A	TGG ACT ATA GCC AGA TAG AGC T	18
RTFL18A	AAG CGA AGA CCT CCT CCT CGA	19
TFLEF1	AGT TCG GCA GCC TCC TCC ACG A	20
TFLER1	TCC AAG GAA AGC CTG AGG TCT T	21
TFLSF1	AAG CTC GCC ATG GTG AAG CTC TT	22
RTFLA	TCG GAG ACG AGT TGG TAG AGG T	23
FTFLB	ACC TCT ACC AAC TCG TCT CCG A	24
RTFLC	AGA GGA CGA AGC CCA CGA A	25
RTFLD	AGG AGG TAG GCG AGG AGC AT	26
FTFLE	ATG CTC CTC GCC TAC CTC CT	27
FTFLF	TCG AGG AGG AGG TCT TCG CTT	28
RTFLG	AGC TCT ATC TGG CTA TAG TCC A	29
FTFLH	ATA GGC TCT CCC AGG AGC TT	30
RTFLI	AAG AGC TTC ACC ATG GCG AGC TT	31
RTFLJ	TTC CCC TGG AGG CGT TTC TGA	32
RTFLK	AAA GAC CAC GAA GAC GGC CTT	33
FTFLL	AAG GCC GTC TTC GTG GTC TTT	34
FTFLM	AAG GAG TGG GGA AGC CTG GAA	35
RTFLN	TTC CAG GCT TCC CCA CTC CTT	36
RTFLO	TTC TTC CGA AGA GGG TTT CCA	37
RTFLP	GCG TCC AGG AGC GCC CTG AGG A	38
FTFLQ	CCT CAG GGC GCT CCT GGA CGC CA	39
FTFLR	TTC GTC CTC TCC CGC CCC GA	40
FTFLS	CCA ACC TGC AGA ACA TCC CCG T	41
RTFLT	GGT GTG GAT GTC CTT CCC CT	42
FTFLU	CCC TGC CGT TTA GAG GAA GTT CAA G	43
RTFLV	CTT GAA CTT CCT CTA AAC GGC AGG G	44
RTFLW	ACC CGG CCT TTG GGT TCA AAG A	45
FTFLX	TCT TTG AAC CCA AAG GCC GGG T	46
RTFLY	TTC CCG TGC TCC TTC CGC TC	47
FTFLZ	CTC GCC TTC CTC GTG CCC TT	48
5'lac PCR	GCT TCC GGC TCG TAT GTT GTG TG	49
TFL-SDM-1	GGA AAG CCT GAG GTC TTC CAT AGC TGT TTC CTG TGT GAA ATT GTT ATC CGC TCA CAA TTC CAC ACA ACA T	50
TFL-SDM-3	ACC CGG CCT TTG GGT TCA AAG AGC GGA ACG ATC GCC TCC ATA GCT GTT TCC TGT GTG AAA TTG TTA TCC GCT CAC AAT TCC	51

EXAMPLE 6

Construction and Expression of a Full-Length *T. flavus* DNA Pol I Clone and Purification of Full-length Recombinant *T. flavus* DNA Pol I

An expression vector containing the full-length *T. flavus* DNA polymerase I gene was constructed as described below, utilizing plasmid p51E9, which contains the 5' portion of the gene, and plasmid p21E10, which contains a 3' portion of the gene that overlaps the 5' portion contained in p51E9. FIGURES 1A and 1B are provided to illustrate steps in the construction of expression vectors of this invention, and are not intended to be a scale representation of clone inserts, or to contain a complete restriction map of clones depicted therein for enzymes shown.

Referring to FIGURE 1A, clone p51E9 which carries the 5' portion of the Tfl DNA pol I gene, was digested with BamHI and a 3.7 kb digestion product was subcloned into the BamHI cloning site of pTZ18U to produce recombinant plasmid, p51B4, which was characterized as containing about 1.5 kb of DNA upstream of the DNA pol I start codon contiguous with the 5' region of the Tfl DNA pol I gene extending to the BamHI site in the 2-4 fragment. Plasmid p51B4 was then digested with XbaI, and a 2.5 kb digestion product was subcloned into the XbaI site of pTZ18U to create plasmid p51X16, which contained only approximately 0.3 kb of DNA upstream of the DNA pol I start codon.

Next, plasmid p21EHc (a subclone of p21E10 described above) was digested with BamHI and SalI. The 3.7 kb fragment containing the 3' region of the Tfl DNA pol I gene, beginning with the BamHI site in the 2-4 fragment, was isolated and subcloned into pTZ18U that had been digested with BamHI and SalI to create clone p21BHc. Clone p21BHc was digested with EcoRV and BamHI and the 1.3 kb fragment containing the 3' region of Tfl DNA pol I was ligated into pTZ18U that had been digested with BamHI and HincII, yielding p21BRV2.

Plasmid p51X16 was digested with BamHI and the 2.5 kb BamHI insert was isolated. Plasmid p21BRV2 was linearized with BamHI and ligated to the BamHI fragment of p51X16. The resulting clones were designated pTFL 1.3 and pTFL 1.4. The integrity of the Tfl DNA pol I gene in clone pTFL 1.4 was verified by DNA sequence analysis using the primer RTFLG (Table 2 and FIGURE 4).

Competent *E. coli* DH5αF' were transformed with plasmid pTFL 1.4 (the 1st generation expression clone), from which a Tfl DNA pol I protein was isolated and purified as follows. *E. coli* DH5αF'[pTFL-1.4] were grown in a 50 liter fermentor (10 pounds back pressure, 30 lpm aeration, 200 rpm agitation, at 37°C) in TB medium (Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd ed. (1989): 12 g Bactotryptone, 24 g yeast extract, 4 ml glycerol, 0.1 g $MgSO_4$, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4 per liter), supplemented with 50 μg/ml ampicillin with vigorous aeration at 37°C. At $O.D._{600}$=1.0, IPTG was added to a final concentration of 0.5 mM and the cells were cultured for an additional 2 hrs. The culture was cooled down to 20°C and 100 ml of 100 mM phenylmethyl sulfonyl fluoride (PMSF) in isopropanol was added. After brief mixing, the culture was spun down in a Sharples centrifuge and the pellet (or paste) was stored frozen at -70°C. Fifty grams of cells were thawed in 250 ml of lysis buffer A (20 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 100 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol, 0.5% Nonidet P40, 0.5% Tween 20, 50 μg/ml PMSF, 0.5 μg/ml pepstatin A, 0.5 μg/ml leupeptin). The cell suspension was homogenized twice in a Manton-Gaulin press. Because PMSF is unstable in aqueous solutions, new PMSF was added again to a final concentration of 50 μg/ml after the first and second homogenizations.

The suspension of broken cells was divided into 100 ml aliquots and heated to 65°C for 1 hr. to denature the bulk of *E. coli* proteins, including nucleases, proteases and *E. coli* polymerases. Cell debris and denatured proteins were centrifuged at 6,800 x g for 30 min. and the NaCl

concentration of the supernatant was adjusted to 400 mM. The presence of DNA polymerase activity in the supernatant was confirmed using the standard activity assay described above. Then 10% polyethyleneimine (PEI, pH 7.5) was slowly added to a final concentration of 0.2%. After 30 min. of stirring at 4°C, the suspension was centrifuged (1 hr., 6,800 x g) and the resulting supernatant was diluted with 6 volumes of buffer C (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 5% glycerol, 5 mM β-mercaptoethanol, 0.5% Nonidet P40, 0.5% Tween 20, 50 μg/ml PMSF, 0.5 μg/ml pepstatin A, 0.5 μg/ml leupeptin). Ammonium sulphate was added to 0.55 g/ml and the mixture was stirred slowly overnight at 4°C.

After centrifugation for 2 hrs. at 6,800 x g the supernatant was carefully removed and tested for DNA polymerase activity. The polymerase-containing pellet was dissolved in a total of 80 ml of Buffer A (10 mM KPO_4 pH 7.0, 0.5 mM EDTA, 100 mM NaCl, 5 % glycerol, 5 mM β-mercaptoethanol, 0.5% Nonidet P40, 0.5% Tween 20, 50 μg/ml PMSF). Insoluble material was removed by centrifugation at 6,800 x g for 20 min. The supernatant obtained from this centrifugation (which contains the polymerase activity) was loaded onto a 5 x 50 cm Sephadex G-25 column equilibrated in buffer A to desalt the solution and to remove traces of PEI. The flow rate used on this column was about 200 ml/hr. Fractions of 25 ml were collected and assayed for DNA polymerase activity. The flow-through fractions contained the activity. It was essential to remove all the PEI for efficient adsorption to the next column.

The crude Tfl DNA polymerase described above was applied to a Bio-Rex 70 column (5 x 10 cm) (Bio-Rad) equilibrated in Buffer A. The column was washed with 1.5 l of buffer A and the DNA polymerase was eluted with 4 l of a 0 - 1 M NaCl gradient in buffer A. Fractions of 25 ml were collected and assayed for DNA polymerase activity. Fractions containing DNA polymerase activity (as assayed below) were pooled, concentrated in an Amicon concentrator with a YM30 membrane to about 40

ml and dialyzed against two changes (1 liter each) of antibody column high salt buffer B (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 M NaCl, 0.05% Brij-35) and applied to an immunoaffinity column (1.5 x 8 cm).

The immunoaffinity column was prepared using techniques well-known in the art. First, a mouse is injected with purified DNA polymerase I to provide an immune response; preferred DNA polymerases for generating antibodies are thermophilic DNA polymerases, including those isolated from *Thermus flavus* and *Thermus aquaticus*. A ten week old female BALB/c mouse (Harlan Sprague Dawley, Madison, WI) was immunized by intraperitoneal injection with Taq polymerase (purified from *Thermus aquaticus* ATCC #25104). To prepare the Taq polymerase for injection, Taq storage buffer was removed with a Centricon 30 protein concentrator (Amicon Corp.), and the concentrated protein was diluted with phosphate-buffered saline. For the initial immunization, 40 μg of Taq emulsified with complete Freund's adjuvant was injected. Five booster injections of 40μg Taq polymerase mixed with equal volumes of the Ribi Adjuvant System (Ribi Immunochem Research, Inc., Hamilton, MT) were administered over a 6 month period, with successive intervals between injections of approximately five weeks, 4 weeks, 4 weeks, 12 weeks, and 4 weeks.

Five days after the final booster injection, the mouse was sacrificed and spleen cells were isolated and fused with myeloma cells (myeloma P3X63-AG8.653 (ATCC CRL 1580)) to generate hybridomas, using techniques well-known in the art. See E. Harlow and D. Lane *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor (1988). In particular, fusions were performed in 50% polyethylene glycol and selected in HAT medium. All hybridomas were screened as described below.

The fifth fusion experiment yielded a useful hybridoma, as selected in the following manner. The hybridomas were distributed into 96-well plates. Of 1176 wells filled, approximately 913 showed growth. To

initially screen these wells, an ELISA assay was employed. First, polystyrene ELISA plates were coated with *Thermus flavus* (ATCC #33923) DNA polymerase (1 μg/ml Tfl DNA pol I (MBR lot 20229) in 100 mM Tris-HCl, pH 8.5/0.05% NaN_3). Five microliter samples of supernatant from each culture were diluted into 95 μl of Tris-buffered saline, pH 8.5/0.05 % Triton X-100 (TBST) and then incubated in the coated ELISA plates for 2 hours at room temperature. The plates were then washed with TBST. To detect positive anti-Tfl DNA polymerase cross-reactivity, a commercially available, peroxidase-conjugated goat anti-mouse IgG, γ chain specific (Jackson ImmunoResearch, West Grove, PA), was diluted 5000-fold in TBST, added to the ELISA plates, and incubated for 1 hour at room temperature. Positive cross-reactivity was detected colorimetrically with 3-methyl-2-benzothiazolinonehydrazone/3-dimethylaminobenzoicacid/hydrogenperoxide.

Supernatants from wells that tested even weakly positive by ELISA were further screened by immunoprecipitation of both Tfl and Taq DNA polymerases using techniques well known in the art. See Harlow and Lane, *supra*. The immunoprecipitation assay employed relies on the presence of protein A (which binds IgG) on the surface of *Staphylococcus aureus* (SAC, Sigma Chemical Co., St. Louis, MO). Since protein A does not bind strongly to a common subclass of mouse IgG, IgG_1, but does bind rabbit IgG strongly, a pellet of centrifuged SAC cells was first treated with rabbit anti-mouse IgG antibodies. The pellet from 10 μl of a 10% suspension of these cells was then incubated with 20 μl of hybridoma culture supernatant for one hour at room temperature. The resultant SAC cells were centrifuged, washed, and resuspended in diluted Taq or Tfl polymerase. The polymerase enzyme-cell suspensions were incubated overnight at 4°C and centrifuged. The resultant supernatant was removed and tested for depletion of polymerase activity using a standard radiochemical assay.

One hybridoma, designated hybridoma 7B12, tested strongly in the ELISA assay and immunoprecipitated both Taq and Tfl DNA polymerases

(70-99% depletion in polymerase activity). More particularly, in a series of immunoprecipitations in which the polymerase concentration (Taq or Tfl polymerase) was varied, the results shown in Table 2B were obtained.

TABLE 2B
IMMUNOPRECIPITATION RESULTS

Trial	Polymerase	Source of Monoclonal antibody	Depletion of polymerase activity
2*	0.04 units Tfl polymerase	hybridoma 7B12 supernatant, 20 μl	>99%
3	0.09 units Tfl polymerase	hybridoma 7B12 supernatant, 20 μl	80%
3	0.27 units Taq polymerase	hybridoma 7B12 supernatant, 20 μl	79%
4	0.30 units Tfl polymerase	1.375 μg purified IgG from hybridoma 7B12	99%

*Trial 1 was unsuccessful due to an excess of polymerase enzyme relative to the amount of antibody.

In control immunoprecipitation experiments in which six anti-*E. coli* DNA polymerase I monoclonal antibodies were employed (25 μl of hybridoma culture supernatant), 91 to 112% of the original Tfl polymerase activity was still detectable in solution (i.e., at most a 9% depletion of Tfl polymerase activity). The monoclonal antibody from hybridoma 7B12 was further characterized and found to neutralize Taq and Tfl polymerase activity at lower temperatures (41°C). This activity assay was performed at 41°C rather than at higher temperatures (70°C) where the enzymes are more active, because the antibody itself denatures at higher temperatures.

Cells from hybridoma 7B12 were cloned three times by limiting dilution until all wells with growth tested positive in the ELISA assay (66/66 wells). The monoclonal antibody of hybridoma 7B12, a mouse IgG (γ1, κ)

antibody, is commercially available from Molecular Biology Resources, Inc., Milwaukee, Wisconsin as Cat. No. 4100-01.

Subsequent experiments produced two additional anti-Taq/anti-Tfl monoclonal antibodies that may be (but have not been) used to affinity-purify DNA polymerase enzymes. In particular, two hybridomas producing anti-Tfl DNA polymerase monoclonal antibodies, formed using spleen cells from a mouse immunized with Tfl DNA polymerase, were identified using the sceening procedures outlined above. In the immunoprecipitation assay, 25 μl of supernatant from these two hybridoma cultures, designated hybridomas 10F10 and 11G4, depleted 92% and 95%, respectively, of the DNA polymerase activity from a solution containing 0.30 units of Tfl DNA polymerase.

The monoclonal antibodies from hybridoma 7B12 were coupled with Emphaze™ resin (3M, St. Paul, MN) as follows. Twenty-five milliliters of antibody solution (2 mg/ml in 0.6 M sodium citrate, 0.05 M sodium chloride, 0.05 M HEPES pH 8.6) was added to 1.25 g of Emphaze™ resin and allowed to react for 2 hrs at room temperature. Ethanolamine (1 ml of a 3 M solution, pH 9.0) was then added to quench unreacted azlactone functional groups and incubated for 1 hr. at room temperature, then overnight at 4°C. The resin was washed with and stored in PBS with 0.05% sodium azide.

The immunoaffinity column for the DNA polymerase purification was prepared with about 10 ml dead volume of the resin and washed with 300 ml of antibody column high salt buffer B (20mM Tris-HCl pH 7.5, 0.5 mM EDTA, 0.5 M NaCl, 0.05% Brij - 35). The Tfl DNA polymerase enzyme was eluted with 10 mM triethylamine (pH 11.6). Fractions (5 ml each) were collected into tubes with 0.01 volumes of 1 M HEPES. Those fractions containing the DNA pol I enzyme were identified by activity assay, pooled, and dialyzed against storage buffer S (50% glycerol, 50 mM Tris-HCl, pH 7.5 at 23°C, 5 mM DTT, 0.1 mM EDTA, 0.5% Tween 20, 0.5% Nonidet P40). The final product was stored at -20°C.

The above purification procedure yielded about 60,000 units of purified *T. flavus* DNA polymerase I from 50 g of *E. coli* [pTFL-1.4] cells, which is equivalent to 1,200 units/g of cells.

The protein concentration was determined by the method of Lowry using a modification of the Sigma (St. Louis, MO) Protein Assay Kit (Cat. No. P5656) with Bovine Serum Albumin as a standard. Both standard and sample were precipitated with TCA prior to the protein analysis. Using the standard activity assay, the DNA polymerase specific activity was calculated to be 79,500 U/mg protein for the recombinant Tfl holoenzyme purified as described.

EXAMPLE 7

Construction and Expression of a High-Yield, Full-Length *T. flavus* DNA Pol I Clone and Purification of Recombinant *T. flavus* DNA Pol I Holoenzyme

To increase expression of the DNA Tfl pol I gene and to increase the yield and DNA polymerase specific activity of recombinant Tfl DNA pol I, the lacZ promoter was fused directly to the ATG start codon of the Tfl DNA pol I gene using site-directed mutagenesis, the resultant improved expression plasmid was expressed, and the recombinant DNA pol I was purified using a modified procedure.

Site-directed mutagenesis of single-stranded uracil-(U-) containing DNA from p51X16 was performed using the oligonucleotide TFL-SDM-3 (Table 2). Single-stranded U-containing DNA was prepared according to the protocol provided by Bio-Rad (Hercules, CA) in their Mutagenesis Kit. The new clone, p51X16M1, had the lacZ promoter fused to about 2 kb of the 5' portion of the Tfl DNA pol I gene (FIGURE 1A). Plasmid p51X16M1 was digested with BamHI and HincII and ligated to the 1.3 kb BamHI/EcoRV fragment isolated from p21BHc, which provided the 3' region of the Tfl DNA pol I gene. The resulting plasmid, pTFLRT4 (ATCC

Accession No. 69633), was used to transform *E. coli* DH5αF'IQ (Life Technologies, Grand Island, NY), generating the 2nd generation expressing clone (FIGURE 1A). The presence and integrity of the Tfl DNA pol I gene in the insert of pTFLRT4 was confirmed by DNA sequence analysis using primers RTFLJ, RTFLG, FTFLB, and FTFLE as set out in Table 2.

E. coli DH5αF'IQ transformed with pTFLRT4 were grown in a 250 liter fermentor in TB medium supplemented with 50 μg/ml ampicillin. At $O.D._{600}$ = 0.7, expression of the plasmid was induced by the addition of IPTG to a final concentration of 0.5mM and the cells were cooled down to 20°C, harvested three hours later, and stored at -70°C until use.

Five hundred grams of induced cells were thawed and suspended in 2500 ml of lysis buffer A (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 100 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol, 0.5% Nonidet P40, 0.5% Tween 20, 50 μg/ml PMSF, 0.5 μg/ml pepstatin A, 0.5 μg/ml leupeptin).

The cell suspension, to which lysozyme was added to a concentration of 0.5 mg/ml, was homogenized in a Manton-Gaulin press. Fresh PMSF again was added to the lysed cells to a final concentration of 50 μg/ml. The suspension of lysed cells was divided into 300 ml portions, heated to 65°C for 1.5 hrs., and centrifuged for 30 min. at 6,800 x g.

Following this centrifugation, the resulting supernatant was adjusted to an additional NaCl concentration of 400 mM and 10% PEI, pH 7.5, was added to a final concentration of 0.2%. After 1 hr. of stirring at 4°C, the suspension was centrifuged (1 hr., 6,800 x g) and the resultant supernatant was precipitated with ammonium sulphate. After centrifugation for 2 hours at 6,800 x g the resultant pellet was resuspended with 200 ml Buffer A and applied to a Bio-Rex 70 column (5 x 10 cm) (Bio-Rad). The column was washed with 1.5 l of buffer A and the DNA polymerase protein was eluted with 4 l of a 0-1 M NaCl gradient in buffer A. Fractions of 25 ml were collected, and the peak fractions were pooled and dialyzed against two

changes (2.5 l each) of antibody column high salt buffer B and applied to an immunoaffinity column (1.5 x 8 cm) prepared as described above. After washing the immunoaffinity column with 250 ml antibody column high salt buffer B, the enzyme was eluted with 10 mM triethylamine (pH 11.6). Fractions of 5 ml were collected and the peak fractions were dialyzed against storage buffer S. This procedure yielded about 2,000,000 units of purified *T. flavus* DNA polymerase from 500 g of *E. coli* [pTFLRT4] cells, or about 4,000 units/g of cells as measured using the standard assay described above. The calculated DNA polymerase specific activity was 217,600 U/mg for this preparation of Tfl holoenzyme.

The N-terminal amino acid sequence from recombinant DNA pol I holoenzyme isolated from *E. coli* [pTFLRT4] was determined as Met-Glu-Ala-Ile-Val-Pro-Leu-Phe-Glu-Pro. This sequence matches the amino terminal sequence deduced by translation of the *T. flavus* DNA pol I gene sequence (FIGURE 2), indicating that the translation starts at the predicted position. Unlike the native holoenzyme studied, no blockage of the terminal methionine in the cloned holoenzyme was observed.

EXAMPLE 8

Cloning and Expression of the Exo⁻ Fragment of *T. flavus* DNA Polymerase I

Expression studies using plasmids p21E10 and p21EHc were performed because these plasmids contain the 3' two-thirds of the DNA polymerase I gene fused to the lacZ operator/promoter. As deduced from the DNA sequence, the first amino acid encoded by the insert of plasmid p21E10 corresponds to Glu_{239} in FIGURE 2 (circled). It was hypothesized that the insert in p21E10 would encode a fragment of DNA polymerase I lacking the exonuclease domain (the exo⁻ fragment) due to the absence of the 5' one-third portion of this gene.

From translation of sequence information obtained from the 5'-end of p21E10 using primer RSP (Table 2), it was concluded that the insert encoding the 3' two-thirds of the Tfl DNA pol I gene was out-of-frame. It was assumed that the same out-of-frame fusion was present in p21EHc. However, in spite of frame shift some heat-stable DNA polymerase activity was obtained from the clone harboring p21E10.

The ATG start codon of lacZ was brought in frame with the TFL DNA polymerase exo fragment in p21EHc using site-directed oligonucleotide mutagenesis (FIGURE 1B). A mutagenic oligonucleotide TFL-SDM-1 was designed (Table 2), part of TFL-SDM-1 having homology to nucleotides 1015-1032 in FIGURE 4, the other part having homology to the vector. Single-stranded U-containing DNA was prepared by standard procedures and the chemically synthesized oligonucleotide TFL-SDM-1 was used to obtain site-directed changes in the newly synthesized DNA. This DNA was used to transform competent *E. coli* DH5αF'. Several transformants were selected and grown up for plasmid analysis. Of these, sequence analysis was performed on four clones using the [γ^{32}P] end-labeled primer "5'lac PCR" (Table 2) (Synthetic Genetics). The clones with the DNA polymerase gene fragment in the proper reading frame (which includes the ATG from the lacZ coding sequence, followed by "GAA GAC..." derived from the Tfl DNA pol I gene - see FIGURE 2, nucleotides 1015-1020 et seq.) were included in expression studies. Overexpressed recombinant protein was then isolated and purified from *E. coli* transformed with one of the clones, p21EHcM1.1 (ATCC Accession No. 69632) by following the procedures outlined below.

E. coli DH5αF' [p21EHcM1.1] was grown in a 50 liter fermentor in TB medium (Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd ed. (1989)) supplemented with 50 μg/ml ampicillin with vigorous aeration at 37°C. At $O.D._{600}$ = 1.0, IPTG was added to final 0.5 mM concentration and cells were cultured for an additional 2 hours. The culture

was cooled down to 20°C and 100 ml of 100 mM PMSF in isopropanol was added. After brief mixing, the culture was spun down in a Sharples centrifuge and stored frozen at -70°C.

Fifty grams of *E. coli* [p21EHcM1.1] were thawed in 250 ml of lysis buffer A (20 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 100 mM KCl, 10 mM $MgCl_2$, 5% glycerol, 5 mM β-mercaptoethanol, 0.5% Nonidet P40, 0.5% Tween 20, 50 μg/ml PMSF, 0.5 μg/ml pepstatin A, 0.5 μg/ml leupeptin). The cell suspension was homogenized twice in a Manton-Gaulin press. After the first and second passes, fresh PMSF was added again to a new, final concentration of 50 μg/ml. The suspension of broken cells was divided into 100 ml portions and heated to 65°C for 1 hr. Cell debris and denatured proteins were centrifuged at 6,800 x g for 30 min. and the supernatant was adjusted to an additional NaCl concentration of 400 mM. Then 10% PEI, pH 7.5, was slowly added to a final concentration of 0.2%. After 30 min. of stirring at 4°C, the suspension was centrifuged (1 hr., 6,800 x g) and the supernatant was concentrated on a YM30 membrane to 100-120 ml. The concentrate was run through a 5 x 50 cm Sephadex G-25 column equilibrated in buffer A, as described in Example 6. The crude Tfl exo$^-$ fragment was applied to a Procion-Red Sepharose column (5 x 10 cm). The column was washed with 1.5 liters of buffer A and the DNA polymerase fragment was eluted with 4 liters of a 0-1.5 M NaCl gradient in buffer A. Fractions of 25 ml were collected and the fractions with DNA polymerase activity were dialyzed against two changes (1 liter each) of antibody column high salt buffer B (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 M NaCl, 0.05% Brij-35) and applied to an immunoaffinity column (1.5 x 8 cm). After washing the column with 250 ml of the same buffer, the enzyme was eluted with 10 mM triethylamine (pH 11.6) and treated as described above. In general, about 300,000 units of purified *T. flavus* exo$^-$ fragment were obtained from 50 g of *E. coli* [p21EHcM1.1] cells (6,000 units/g).

The protein concentration was determined as described above. The calculated DNA polymerase specific activity for the Tfl exo⁻ fragment was 600,000 U/mg.

Once the Tfl exo⁻ fragment was cloned and expressed the N-terminal amino acid sequence was determined. About 50 μg of the purified enzyme was separated using SDS-PAGE and blotted onto PVDF membrane as described for the holoenzymes. The major band was excised and subjected to sequence analysis. The chromatogram of the sequencer indicated the presence of a major and a minor sequence. The minor sequence represents the major sequence shifted by one amino acid. The major sequence reads: Leu-Glu-Arg-Leu-Glu-Phe-Gly-Ser-Leu-Leu-His-Glu-Phe-X-Leu-Leu-X-Ala-Pro-Ala (where X represents an amino acid whose identity was uncertain from the chromatogram). The minor sequence has the amino acid sequence: Glu-Arg-Leu-Glu-Phe-Gly-Ser-Leu-X-His-Glu-Phe-Gly-X-X-Pro-X-X-Ala-Pro.

The major sequence is identical to the amino acid sequence deduced from the recombinant Tfl exo⁻ fragment DNA sequence, except for the lack of 37 N-terminal amino acids, including the N-terminal methionine. SEQ ID NO: 3 and 4 contain the DNA sequence and the deduced amino acid sequence of the Tfl exo⁻ fragment, as expected from construct p21EHcM1.1. The loss of the 37 N-terminal amino acids may be due to processing of the exo⁻ fragment in the *E. coli* host. SEQ ID NO: 5 contains the amino acid sequence of the major band exo⁻ fragment, as deduced from the N-terminal amino acid sequence of the purified exo⁻ fragment and from the DNA sequence of plasmid p21EHcM1.1. The minor sequence presented here is the Tfl exo⁻ fragment lacking both the N-terminal methionine and the next 37 amino acids. Although the amount sequenced in the minor species was small there was good correlation with the deduced amino acid sequence, except for the proline at position 16, that was expected to be glutamic acid.

EXAMPLE 9

Characterization of *T. flavus* DNA Polymerase I Exonuclease Activities

The purity and molecular weight of the *T. flavus* DNA polymerase and the Tfl exo⁻ fragment were estimated by SDS-polyacrylamide gel electrophoresis using the Pharmacia PhastSystem (Piscataway, NJ). FIGURE 9 shows the purity of the holoenzyme and the Tfl exo⁻ fragment, which were separated on a 12.5% SDS-PAGE gel and stained with silver.

Assays were performed to determine intrinsic/extrinsic exonuclease, endonuclease, and DNase activities of the DNA polymerase enzyme preparations purified as described above and for *T. aquaticus* DNA pol I holoenzyme (Taq holo) and Stoffel fragment (Stoffel, Perkin Elmer, Foster City, CA, Cat. No. N808-0038), and for *T. thermophilus* holoenzyme (Tth holo) (Molecular Biology Resources, Inc., Cat. No. 1115-01, Milwaukee, WI). The protocols are described below and results summarized in Table 3A.

A 3'→ 5' exonuclease activity assay was performed in a final volume of 10 μl containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 0.15 μg of [3'-^{3}H dCTP and dGTP labeled]λ DNA/Taq I fragments and 5, 10 and 20 units of enzyme. Each sample was overlaid with 10 μl of light mineral oil and incubated at 70°C for 1 hour. The reaction was terminated by the addition of 50 μl yeast RNA and 200μl of 10% TCA. After incubation for 10 min. on ice, the samples were centrifuged for 7 min. in a microcentrifuge. 200μl of supernatant was added to 6 ml of scintillation fluid and counted in a scintillation counter. The results are presented in Table 3A as the slope %-label released per unit of enzyme.

A 5' → 3' assay was performed in a manner identical to the 3' → 5' exonuclease assay, except for the use of [5'-^{32}P] λ DNA/ HaeIII fragments instead of the 3'-labeled substrate.

Double-stranded and single stranded DNase assays were performed using the protocol for the 3' → 5' exonuclease assay, except for the use of [^{32}P] λ DNA instead of the 3'-labeled substrate. The DNA was treated for 3 min. at 100°C and immediately chilled on ice prior to assaying for single stranded DNase activity.

An assay for endonuclease activity was performed as follows. The reagents (final concentrations of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol), 0.5 μg pBR322, less enzyme/H_2O, were mixed and kept on ice. The required amount of H_2O and 10 μl mineral oil were added to each tube. The reaction was started with the addition of 5, 10 or 20 units of enzyme; the final reaction volume was 10 μl. The samples were incubated at 70°C for 1 hour. Two μl of a solution containing 0.25% bromophenol blue, 1 mM EDTA, and 40% sucrose was added to the reaction, and after a short centrifugation, 6 μl of the bottom layer was removed and electrophoresed on 1.5% agarose gels in 1 x TBE. The mobility change from the supercoiled to the linear form of pBR322 was recorded.

TABLE 3A

Enzyme	3'→5' exo-nuclease	5'→3' exo-nuclease	ss DNase	ds DNase	Endo-nucle-ase
Tfl holo (r)	0.19	0	0.66	0	0
Tfl holo (n)	0.04	0.01	0	0	0
Tfl exo⁻	0.03	0.002	0	0	0
Taq holo	0.031	0.09	0	0	0
Stoffel	0	0.01	0.28	0.1	0
Tth holo	0.07	0.04	0.2	0	0

The values for 3'→ 5' exonuclease activity and for 5'→ 3' exonuclease activity are low for all DNA polymerases tested. The differences in exonuclease and DNase activities between naturally occurring and recombinant Tfl holoenzyme are not believed to be statistically significant.

EXAMPLE 10

Comparison of the *T. flavus* and *T. aquaticus* DNA Polymerases

Biological properties of native *T. flavus* DNA pol I (nTfl Holo, lot #30419; Molecular Biology Resources, Inc., Cat. No. 1112-01, Milwaukee, WI); recombinant *T. flavus* DNA pol I holoenzyme (rTfl Holo) purified from *E. coli* [pTFLRT4]; *T. flavus* DNA pol I exo⁻ fragment (Tfl exo⁻) purified from *E. coli* [p21EHcM1.1]; *T. aquaticus* DNA polymerase I (native Taq or recombinant AmpliTaq) holoenzyme; and the AmpliTaq DNA polymerase Stoffel fragment were compared using a number of protocols described below.

The molecular weights and purities of the preparations of the various enzymes were estimated by acrylamide gel electrophoresis utilizing the Pharmacia PhastSystem (Piscataway, NJ) for electrophoresis and silver staining. A comparison of the apparent molecular weights estimated from 7.5% and 12.5% acrylamide gels and the calculated molecular weights derived from available sequence data is given in Table 3B. The apparent molecular weight of the holoenzymes using either acrylamide concentration was less than the calculated molecular weights. A purity of greater than 95% was estimated for all DNA polymerases analyzed: i.e. Tfl and Taq holo enzymes, Tfl exo⁻ fragment and Stoffel fragment.

TABLE 3B Apparent Mol. Weight			
Enzyme	7.5% gel	12.5% gel	Calculated Mol. Weight
Tfl holoenzyme*	80,000	84,000	93,969
Recombinant Tfl exo⁻ fragment (recomb.)	59,000	59,000	62,979
Taq holoenzyme	82,000	85,000	93,904
Stoffel	60,000	61,000	61,000

* native and recombinant

Using the Pharmacia PhastSystem, the polymerases and standards were subjected to isoelectric focusing. The experimentally derived pI values of the samples, including samples of *E. coli* DNA pol I holoenzyme (*E. coli* pol I) and Klenow fragment, were compared to values calculated from derived amino acid sequence information. The results are given in Table 4.

TABLE 4 pI Values		
Enzyme	Calculated pI	Measured pI
nTfl Holo	6.23	6.25
rTfl Holo	6.23	6.43
A&V Tfl*	5.73	(not available)
Tfl exo⁻	6.37	5.94
Taq Holo	6.00	6.14
Taq Stoffel	5.93	5.83
E. coli pol I	5.29	5.12
Klenow	5.60	5.75

*Purported *T. flavus* DNA pol I protein sequence published by Akhmetzjanov and Vakhitov.

The relative DNA polymerase activities of the enzymes were assayed at 70°C at different pH values. The pH of selected buffers were adjusted at 23°C, to permit direct comparison to published results. Table 5A shows the measured pH values at 70°C for 1x buffers which first had been titrated at 23°C. Unless otherwise indicated, pH values reported herein were adjusted at about 23°C.

TABLE 5A
Change of pH as a function of temperature

No.	Buffer	pH at 23°C	pH at 70°C
1.	PIPES-NaOH	6.0	5.5
2.	PIPES-NaOH	6.5	6.0
3.	Tris-HCl	7.5	6.4
4.	Tris-HCl	8.0	7.0
5.	Tris-HCl	8.5	7.4
6.	Tris-HCl	9.0	8.0
7.	Tris-HCl	9.5	8.6
8.	Triethylamine-HCl	9.5	8.9
9.	Triethylamine-HCl	10.0	9.15

The activity assays were performed in a 100 μl (final volume) reaction mixture, containing 0.1 mM dCTP, dTTP, dGTP, [α^{33}P]dATP, 0.3 mg/ml activated calf thymus DNA and 0.5 mg/ml BSA in a set of buffers containing: 50 mM KCl, 1 mM DTT, 10 mM $MgCl_2$ and 50 mM of one of three buffering compounds: PIPES, Tris or Triethylamine. Three dilutions (20, 40 or 80 U/μl) of each polymerase enzyme were prepared, and 5 μl of a dilution was added to the reaction mixture, followed by incubation at 70°C for 30 min. The experiment was performed twice, each time using duplicate samples. FIGURE 5 graphically depicts the relative activities of the enzymes

studied, calculated as the ratio of counts per minute (corrected for background and enzyme dilution) at a given pH to counts per minute at the maximum value for that enzyme. The optimal ranges (>80% activity) for the five enzymes tested are provided in Table 5B.

TABLE 5B
Optimal pH ranges (as titered at 23°C)

Enzyme	pH
Native Tfl holoenzyme	9.5-10.5
Recombinant Tfl holoenzyme	9.5-10.5
Tfl exo⁻ fragment	7.5-9.8
Stoffel fragment	7.5-9.8
Ampli Taq	7.5-9.3

These values are about 1 pH unit higher than for buffers measured at 70°C (see Table 5A).

The pH protocol described above was modified to determine the influence of $MgCl_2$ concentration on the activities of the DNA polymerases. The reaction buffers included 50 mM Tris-HCl pH 8.3 (23°C) and $MgCl_2$ concentrations from 0.36 to 50 mM. Three independent experiments were performed and curves were constructed (FIGURE 6A) showing the relative activity of Tfl exo⁻ fragment, Tfl holoenzyme (native and recombinant), and Taq Stoffel fragment. The higher limit for the Stoffel fragment was extrapolated. The optimal ranges (>80% activity) are 1.3-13 mM $MgCl_2$ for the Tfl exo⁻ fragment, and 2.3-33 mM $MgCl_2$ for the Stoffel fragment. The recombinant and the native Tfl holoenzyme showed greatest activity at 50 mM $MgCl_2$.

The above protocol was modified to determine the influence of $MnCl_2$ concentration on the activities of the DNA polymerases (in the absence of magnesium ions). The reaction buffers included $MnCl_2$ concentrations

from 0.1 to 20 mM. Due to the precipitation of oxidation products (MnO_2) of $MnCl_2$, the reaction buffers were prepared just prior to the assay. The pH of the buffer was adjusted to pH 8.7 before the addition of a 1 M stock solution of $MnCl_2$. The pH was finally adjusted to 8.3 at 23°C. Three independent experiments were performed and a curve was constructed (FIGURE 6B) showing relative activity of the enzymes. The optimal ranges for the four enzymes tested are 2.1-11 mM $MnCl_2$ for the Tfl exo⁻ fragment, 4-20 mM $MnCl_2$ for the Stoffel fragment and 0.8-4 mM $MnCl_2$ for the recombinant and native Tfl holoenzymes.

The thermostability and temperature optimum of the polymerase enzymes were determined by incubating 10 units of enzyme for 30 min. at 23, 37, 60, 65, 70, 75, 80, and 90°C, in 100 μl of buffer used for the determination of polymerase activity (including 50 mM Tris-HCl pH 8.3 (23°C) and 1.5 mM $MgCl_2$) in a DNA polymerase activity assay as described above. The polymerase activity was then determined by acid precipitation of the polymerization product as described above. FIGURE 7A depicts the percent relative activity, calculated as described above. The temperature optima were 70-75°C for the Stoffel and Tfl exo⁻ fragments and 80°C for the native and recombinant Tfl holoenzymes. At 90°C there was 14%, 6% and 8% of the activity left in the Tfl holoenzymes, the Stoffel fragment, and the Tfl exo⁻ fragment, respectively.

The PCR half lives of the enzymes were determined in 100 μl PCR reactions, performed in duplicate, substituting the appropriate buffer in the PCR cocktail prepared for each individual enzyme. The cocktail for the Tfl exo⁻ fragment contains 1 x Tfl pol buffer (50 mM Tris-HCl, pH 9.0 at 23°C, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$), 200 μM of each dNTP, 0.5 μM of primer FTFL2 and primer RTFL4, and 15 ng of *T. flavus* genomic DNA. The buffers for other enzymes tested were as follows: Taq pol I (1 x Taq pol buffer: 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$); Tfl DNA pol I holoenzyme (1 x Tfl pol buffer); and Stoffel fragment (1 x Stoffel buffer: 10

mM Tris-HCl, pH 8.3, 10 mM KCl and 2.5 mM $MgCl_2$). Duplicate samples were denatured at 95°C for 5 min. and held at 72°C until 10 units of enzyme were added, and the samples were then cycled 0, 20, 25, 30, 35, 50 and 100 times as described in Example 3. The samples were analyzed on 1.2% agarose gels using ethidium bromide to visualize the presence of specific PCR product. The expected length of PCR product was about 800 bp. Reactions containing Taq DNA pol I, Stoffel and Tfl exo⁻ fragments had visible product after 20 cycles, whereas reactions with Tfl holoenzyme showed product only after 30 cycles. The Tfl exo⁻ fragment synthesized more product than the Stoffel fragment (FIGURE 7B). In general, there was some background, very likely because of the large amount of enzyme in the reaction. This background was not observed when 1 to 5 units of Tfl exo⁻ fragment were used in a 35 cycle regimen. (FIGURE 7B, Right lane.) The polymerase activity in each tube was also determined as described above following completion of the PCR cycling, and the result plotted in a enzyme cycling stability curve (FIGURE 8). The half life was estimated to be: 25 cycles for both the Taq holoenzyme and Stoffel fragment, 20 cycles for the Tfl holoenzyme, and 16 cycles for the Tfl exo⁻ fragment.

EXAMPLE 11

DNA Sequencing with *T. flavus* DNA Polymerases

A.

Native and recombinant Tfl holoenzyme, Tfl exo⁻ fragment, AmpliTaq, and Stoffel fragment were employed in the SEQUAL™ DNA Polymerase Sequencing System (CHIMERx) to test their performance in DNA sequencing using ssDNA template and labeled primer.

The primer FSP (Table 2) was end-labeled with T4 kinase and [γ^{32}P]ATP according to the CHIMERx protocol. A 10 μl labeling reaction was prepared containing 0.5 μl primer (10 pmol/μl), 1.0 μl T4 Kinase 10X buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, 1 mM

spermidine), 3.0 μl [γ-^{32}P] ATP (6000 Ci/mmol, 10μCi/μl), 0.5 μl T4 kinase (15 U/μl), and 5.0 μl H_2O. The labeling reaction was incubated at 37°C for 10 min., and the kinase was inactivated by incubation at 65°C for 10 min.

The sequencing reactions for native and recombinant Tfl holoenzyme and the exo fragment were set up using 2-5 units of enzyme, according to CHIMERx conditions. Briefly, a reaction cocktail was prepared containing 16.0 μl ssM13mp18 DNA (approx. 1 μg), 5.0 μl Sequal 5x buffer (250mM Tris-HCl, pH 9.5, 12.5 mM $MgCl_2$), 1.0 μl labeled primer (0.5 pmol/μl), and 2-5 units of enzyme. Four d/ddNTP mixtures were also prepared (A mix: 20 μM dATP, 60 μM dCTP, 60 μM dGTP, 60 μM dTTP, 300 μM ddATP; C mix: 60 μM dATP, 20 μM dCTP, 60 μM dGTP, 60 μM dTTP, 150 μM ddCTP; G mix: 60 μM dATP, 60 μM dCTP, 20 μM dGTP, 60 μM dTTP, 30 μM ddGTP; T mix: 60 μM dATP, 60 μM dCTP, 60 μM dGTP, 20 μM dTTP, 400 μM ddTTP). The sequencing reactions were performed by mixing 5 μl of reaction cocktail with 1 μl of the appropriate d/ddNTP mixture and heating the reaction tube at 65°C for 10 min. After this incubation 3 μl of stop solution (EDTA/DTT/Bromophenol blue/xylene cyanol) were added and the reactions were placed on ice.

AmpliTaq reaction cocktail was prepared by using the 10x Reaction Buffer provided with the Cycle Sequencing Kit, which contains 2 units of enzyme in a final volume of 20 μl. Stoffel fragment reaction cocktail (20 μl) contained 4 μl of 5x Stoffel fragment reaction buffer, 2 μl of 25 mM $MgCl_2$, both provided with the enzyme (Perkin Elmer), and 2 units of enzyme. Both cocktails included 1μg of ssDNA template and 1 μl of labeled primer FSP. For both enzymes, sequencing reactions were performed by mixing 5μl reaction cocktail with 1μl d/ddNTP mixtures and incubating for 10 min. at 65°C. Three microliters of stop solution were then added to the reactions, and the reactions were placed on ice.

The reactions were heated at 90°C for 5 min. just before loading onto a 6% sequencing gel. One microliter of each sample was loaded

and electrophoresed at 3000 volts for 1.5 hours. The gel was autoradiographed and analyzed. FIGURE 10A photographically depicts a portion of a sequencing gel showing the same DNA sequence for all enzymes used, except for the native *T. flavus* DNA pol I holoenzyme control. Very little background was observed when the Tfl exo⁻ fragment, Tfl holoenzyme and AmpliTaq were used. The Stoffel fragment had more ghost bands than the other enzymes. However, no attempt had been made to optimize the reaction conditions for the Stoffel fragment.

B.

To demonstrate the utility of the recombinant Tfl holoenzyme and the Tfl exo⁻ fragment in *cycle* sequencing with single-stranded DNA template, these enzymes were substituted into the SEQUAL™ DNA Polymerase and Cycle- SEQUAL™ Sequencing System (CHIMERx) and the protocols provided were followed.

The labeling protocol described above was repeated to create end-labeled primer. A 22 μl reaction cocktail was then prepared containing approx. 20 ng ssM13mp18 DNA, 5.0 μl 5X Sequal sequencing buffer, 1.0 μl labeled primer (0.5 pmol/μl), balance H_2O. Native or recombinant Tfl holoenzyme or Tfl exo⁻ fragment was then added to the cocktail (0.5 units) and gently mixed. For comparison purposes, two units of AmpliTaq or Stoffel fragment were added to the ssM13mp18 DNA template (20 ng); the manufacturer's reaction conditions for the Perkin Elmer Cycle Sequencing Kit were followed for AmpliTaq, and, for the Stoffel fragment, 4 μl of the Stoffel buffer and 2 μl of the $MgCl_2$ solution provided with the enzyme were used.

The sequencing reactions were performed by mixing 5 μl of reaction cocktail with 1 μl of each d/ddNTP mixture (in separate tubes), adding a drop ($\sim 10\mu$l) of mineral oil to each tube, and placing the tubes into a preheated (94°C) thermal cycler programmed to run the following cycle twenty times: 94°C for 15 seconds (denaturation), 70°C for 60 seconds

(extension). The reactions were cooled to 4°C after 20 cycles until 4μl stop solution were added, and then the reactions were set on ice.

Immediately after heating the reaction mixtures at 90°C for 5 min., one microliter of each reaction mixture was loaded onto a 6% sequencing gel. FIGURE 10B shows that the Tfl exo fragment and recombinant Tfl holoenzyme yield clean sequence data, whereas in the AmpliTaq lanes some ghost bands were observed. The Stoffel fragment, under the conditions used here, did not produce comparable sequencing data.

C.

The utility of recombinant Tfl holoenzyme and Tfl exo fragment for sequencing with internal labeling using double-stranded DNA template was demonstrated in a sequencing reaction in which a [α^{35}S]-dATP labeling protocol and double stranded pUC19 template were used. The experiment was performed as outlined in the SEQUAL™ DNA Polymerase Sequencing System (CHIMERx) with 2 μg of pUC19 dsDNA and 2.5 units of the enzymes.

To promote efficient priming, the pUC19 double-stranded DNA template was denatured by adding deionized H_2O to 18 μl, adding 2 μl of 2M NaOH, and incubating for 5 min. at room temperature. The reaction was neutralized by adding 2 μl of 2M ammonium acetate, pH 4.6, ethanol precipitated, air-dried, and resuspended in 10 μl deionized water.

For each enzyme, a 22.75 μl extension/labeling cocktail was prepared with the 2 μg denatured pUC19 dsDNA, 5.0 μl 5X Sequal buffer, 1.0 μl primer (0.5 pmol/μl), 1.0 μl alpha labeling mix (~45 μM each of dCTP, dGTP, dTTP), 0.25 μl [α-^{35}S] dATP (1000 Ci/mmol), 2.5 units enzyme, balance H_2O. This cocktail was incubated at 65° for 10 min.

Extension/termination reactions were performed by adding 5 μl of extension/labeling cocktail to tubes containing 1 μl of the appropriate d/ddNTP mix, and mixing gently. The reaction tubes were immediately

placed at 65°C for 4 min., stopped by addition of 4 μl step solution, and set on ice.

Each reaction was heated at 90° for 5 min. immediately before loading 1-2 μl onto a sequencing gel. Results are depicted in FIGURE 10C. Native Tfl holoenzyme (not shown) was compared to recombinant holoenzyme and to the Tfl exo⁻ fragment. The bands were comparable for the holoenzymes. The quality of the sequence data is comparable although the signal was weaker when the Tfl exo⁻ fragment was used.

D.

The utility of recombinant Tfl holoenzyme and the exo⁻ fragment for double-stranded sequencing using a labeled sequencing primer was demonstrated by substitution of these enzymes into the SEQUAL™ System which uses 2 μg pUC19 dsDNA and [γ^{32}P]-labeled primer FSP. The reactions were performed according to CHIMERx's protocol. More particularly, the double-stranded template was first denatured as described above, and then sequencing reactions were performed essentially as decribed in part A (substituting the pUC19 denatured dsDNA for ssM13mp18 template). As can be seen in FIGURE 10D, both the Tfl holoenzyme and the Tfl exo⁻ fragment produced good sequence data.

EXAMPLE 12

Polymerase Chain Reaction

The utility of recombinant Tfl holoenzyme and the exo⁻ fragment in PCR was demonstrated as follows. In a 0.5 ml reaction tube 85 μl water, 2 μl 10 mM dNTPs, 10 ml 10 x Tfl Polymerase Reaction Buffer (10 x buffer is 500 mM Tris-HCl, pH 9.0, 200 mM $(NH_4)_2SO_4$, 15 mM $MgCl_2$), 1 μl each of 50 μM primers FTFL11 and RTFL12 (primer set 11-12), 50 μl mineral oil and 1 μl of 15 μg/ml *T.flavus* genomic DNA were combined. After the initial denaturation step (Step 1), 5.5 and 11 units of Tfl exo⁻ fragment, or 5 units of Tfl holoenzyme were added. As a control Taq pol I

in 1 x Taq Polymerase Reaction Buffer (Example 10) was used to amplify the genomic DNA. Amplifications were performed in a MJ Research PTC-100 Cycler with external sensor control. The amplification program was: Step 1: 95°C for 5 min.; Step 2: hold at 72°C; Step 3: 55°C for 45 sec.; Step 4: 72°C for 5 min.; Step 5: 95°C for 15 sec.; Step 6: repeat steps 3-5 thirty-four times; Step 7: 55°C for 45 sec.; Step 8: 72°C for 20 min.; Step 9: hold at 4°C.

The amplification products were separated on 1.2% agarose gels. Primer set 11-12 gave a single amplification product from *T. flavus* genomic DNA. Five units of the Tfl exo⁻ fragment produced a single product: the yield was slightly less than that obtained with Taq polymerase and better than the yield from Tfl holoenzyme.

EXAMPLE 13

Thermal Cycle Labeling With Tfl DNA Pol I

The protocol described in Example 4 was used to demonstrate the utility of recombinant Tfl DNA pol I holoenzyme and the Tfl exo⁻ fragment for thermal cycle labeling (TCL). See co-owned, co-pending U.S. Patent Application Serial No. 08/217,459, filed March 24, 1994, entitled "Materials and Methods for Restriction Endonuclease Applications." PCT Application No. US94/03246, filed March 24, 1994.

Thermal Cycle Labeling (TCL) is a procedure for labeling double-stranded DNA while simultaneously amplifying large amounts of the labeled probe. TCL of DNA requires two general steps: 1) generation of the sequence-specific oligonucleotides by *CviJI*® (Molecular Biology Resources, Milwaukee, WI) restriction of the template DNA; and 2) repeated cycles of denaturation, annealing, and extension in the presence of a thermostable DNA polymerase or a functional fragment thereof which maintains polymerase activity. Optimal results are obtained after 20 such cycles, which is best performed in an automated thermal cycling instrument such as a Perkin-Elmer

Model 480 thermocycler. In conjunction with such an instrument, about 1.5 hr. is required to complete this protocol. If a thermal cycler is not available these reactions may be performed using heat blocks. As few as 5 cycles may yield probes with acceptable detection sensitivities. The generation of sequence specific oligonucleotides for use in this method may also be accomplished using the restriction endonuclease reagent CGase I (Molecular Biology Resources) or the restriction endonuclease Aci I which has as a recognition sequence CCGC.

Non-radioactive labeling of DNA using TCL is accomplished by mixing: 10 pg - 100 ng linearized template, 50 ng *Cvi*JI*-digested primers, 1.5 μl 10X labeling buffer, 2.5 - 5 units thermostable DNA polymerase, 1 μl of 1mM Biotin-11-dUTP (Enzo Diagnostics, New York, New York), 1.5 μl each of dATP, dCTP, and dGTP (2 mM), and 1.0 μl 2mM dTTP. The reaction mixture is brought to a volume of 15 μl with deionized H_2O, overlaid with mineral oil and cycled through 20 rounds of denaturation, annealing and extension. A typical cycling regimen employs 20 cycles of denaturation at 91°C for 5 sec, annealing at 50°C for 5 sec and extension at 72°C for 30 sec. The reaction is then terminated by adding 1 μl of 0.5M EDTA, pH 8.0. The amplified, labeled probe is a very heterogeneous mixture of fragments, which appears as a smear when analyzed by agarose gel electrophoresis.

The performance of recombinant Tfl DNA pol I holoenzyme, Tfl exo^{-} fragment, Taq holoenzyme, and the Stoffel fragment (control) was assayed by substitution of these enzymes for the enzyme provided with the CHIMERx TCL kit (ZEPTO™ Labeling Kit). Five units of each enzyme and biotin-11-dUTP as the label were used. The substrate was pUC19 DNA.

After cycling of the samples the relative efficiency of the labeling reaction was determined by electrophoresis on a 0.7% agarose gel. The ethidium bromide gel staining of amplified DNA shows the characteristic smear for all enzymes used. The efficiency of incorporation was then determined by dot blot analysis.

The hybridized and developed filter showed that the holoenzymes (native and recombinant Tfl, and Taq) can be diluted 1: 10^6 and still generate a visible dot. The samples which were labeled with Tfl exo$^-$ or the Stoffel fragment can clearly be seen after a 1: 10^4 dilution. The 1: 10^5 dilution gave a weak signal when the exo$^-$ fragment was used for the TCL reaction.

Another aspect of the present invention involves a variation of TCL called Universal Thermal Cycle Labeling (UTCL) in which the extension primers are not supplied by CviJI* restriction. Without intending to be limited to a particular theory, one explanation for the mechanism of UTCL is that the Tfl DNA pol I holoenzyme itself may supply endogenous "random" primers for enzymatic extension in a TCL-type reaction. Alternatively, some other explanation accounts for the mechanism of UTCL.

In a UTCL reaction, recombinant Tfl DNA pol I holoenzyme is combined with intact DNA template and is subjected to repeated cycles of denaturation, annealing, and extension. A radioactive- or non-isotopically-labeled deoxynucleotide triphosphate is incorporated during the extension step for subsequent detection purposes. The amplified, labeled probe represents a very heterogenous mixture of fragments, which appears as a large molecular weight smear when analyzed by agarose gel electrophoresis. The utility of recombinant Tfl DNA pol I for Universal Thermal Cycle Labeling is demonstrated by substituting this enzyme in the UTCL protocol described in co-owned, copending U.S. Patent App. Ser. No. 08/217,459, filed March 24, 1994 (Example 12), incorporated herein by reference.

EXAMPLE 14

Reverse Transcription with *T. flavus* DNA Pol I Holoenzyme and exo$^-$ Fragment

RNA-dependent DNA polymerase activity of the Tfl DNA polymerases was analyzed using the following procedure: In a 0.5 ml reaction

tube, 2.5 μl 1 M Tris-HCl, pH 8.3, 5μl of 0.6 M KCl, 5 μl of 0.04 M $MgCl_2$, 17.5 μl of water, 10μl of 2 mM poly rA:dT (the substrate) and 5 μl 5 mM [α-^{32}P]TTP at 10 μCi/ml were combined. After incubation at 55°C for 5 min., the reaction was started by the addition of 5 μl of enzyme (Five DNA-dependent DNA polymerase units per μl). The reaction was allowed to proceed at 55°C for 30 min., and terminated by taking a 40 μl aliquot and adding it to 50 μl of 10% tRNA, 2% sodium pyrophosphate. The samples were precipitated with TCA and the enzyme activity was determined as described above. The RNA-dependent polymerase activity of the native and the recombinant Tfl DNA pol I was determined to be about 6% of the DNA-dependent polymerase activity. When 10 (RNA-dependent DNA polymerase) units of AMV-RT (Molecular Biology Resources, Inc., Cat. No. 1372-01) were compared to 10 (DNA-dependent DNA polymerase) units of Tfl DNA pol I it was found that nTfl DNA pol I possess 2.4% and rTfl DNA pol I 1.6% of the RNA-dependent DNA polymerase activity of AMV-RT. Titration of the $MgCl_2$ and the $MnCl_2$ concentration revealed that the native and the recombinant holoenzymes prefer $MgCl_2$ over $MnCl_2$ for RT activity.

The Tfl exo⁻ fragment has a lower RT activity than the holoenzyme, but has a broader temperature range for activity. First strand cDNA synthesis with the holoenzymes apparently yields a product of the same length as that obtained by using AMV-RT. The recombinant *T. flavus* DNA polymerase I and the exo⁻ fragment both exhibit reverse transcriptase function which can be used in applications such as RT-PCR or cDNA preparation at elevated temperatures.

EXAMPLE 15

Comparison of the Processivity of DNA Polymerase Enzymes

Using a modification of a procedure described by Tabor et al., *J. Biol. Chem. 262:* 16212-16223 (1987), the processivity of native and

recombinant Tfl DNA pol I holoenzyme, Tfl exo⁻ fragment, and Taq DNA pol I holoenzyme were compared. The "processivity" of a DNA polymerase enzyme is a measure of the rate at which the enzyme moves forward along a template while catalyzing DNA synthesis, i.e., a measure of the speed at which DNA polymerization takes place in the presence of the enzyme.

To prepare the assay, a 60 μl reaction cocktail was prepared with 3μg M13 mp18 ssDNA, 12 μl ddATP mix (20 μM dATP; 60 μM each of dCTP, dGTP, and dTTP; 300 μM ddATP), 3.0 μl α-^{33}P labeled forward sequencing primer (3 μg/μl), 12μl 5x reaction buffer (250 mM Tris-HCl, pH 9.5; 12.5 mM $MgCl_2$), balance H_2O. Additionally, dilutions of native and recombinant Tfl holoenzyme, Tfl exo⁻ fragment, and Taq holoenzyme were prepared with appropriate storage buffer to create enzyme solutions of 0.125 and 0.0125 units/μl for the holoenzymes and 0.25 and 0.025 units/ml for Tfl exo⁻ fragment.

To perform the assay, 7.0 μl of the reaction cocktail were mixed with 2.0 μl of diluted DNA polymerase enzyme. By using 0.25 and 0.025 units of Taq, nTfl, and rTfl holoenzyme and 0.5 and 0.05 units of exo⁻ fragment per reaction, reactions containing approximately 1:100 and 1:1000 enzyme molecule: template molecule are obtained. The use of such low polymerase concentrations minimizes the "bumping off" from template by competing polymerase molecules. Reaction mixtures were incubated at 65°C and 3μl samples were removed at 1.0, 2.5 and 6.0 minute time points. Reactions were stopped by adding 1.0 μl stop buffer (EDTA/DTT/BromoPhenol Blue/xylene cyanol), were heated at 90°C for 3 min., and were loaded onto 7.5% polyacrylamide sequencing gels. The gels were electrophoresed until the bromophenol blue dye was about 3/4 down the gel, and an autoradiograph of the gel was taken overnight at -70°C.

With this assay, a highly processive enzyme produces stong, slow-mobility (larger) labeled bands on an autoradiograph, whereas a less processive DNA polymerase produces higher-mobility (smaller) fragments

and/or bands with less intensity. Autoradiographs of the 6 min. incubation/1:100 enzyme:template reactions revealed the exo⁻ fragment produced bands with the most intensity, followed by the rTfl and nTfl holoenzyme, then the Taq holoenzyme. The length of fragments obtained by the four enzymes was very comparable. Autoradiographs from the 1:1000 enzyme:template reaction indicate that processivity (from best to least) is Tfl exo⁻ fragment > Taq holoenzyme > nTfl holoenzyme and rTfl holoenzyme. These results indicate that Tfl exo⁻ fragment has greater processivity than either Tfl holoenzyme (native or recombinant) or Taq holoenzyme.

EXAMPLE 16

Large-Scale Purification of Recombinant Tfl DNA Polymerase I Holoenzyme and Exo⁻ Fragment

Both the recombinant Tfl holoenzyme and Tfl exo⁻ fragment were purified on a large "production" scale by modifying the procedure described above for purifying native Tfl holoenzyme.

Four hundred sixty grams of induced *E. coli* DH5αF'IQ cells transformed with pTFLRT4 (cultured and frozen as described above) were thawed and suspended in 2500 ml of lysis buffer A (20 mM Tris-HCl, pH 8; 0.5 mM EDTA; 7 mM β-mercaptoethanol; 10 mM $MgCl_2$). For Tfl exo⁻ fragment, 787 grams of *E. coli* transformed with p21EHcM1.1 (cultured and frozen as described above) were used. Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.3 mM.

The suspension was then treated with 0.2 g/l of lysozyme (predissolved in lysis buffer) at 4°C for 1 hr. Cells were homogenized twice at 9000 psi in a Manton Gaulin homogenizer, with the suspension chilled to approximately 10°C between passes. New PMSF was added to 0.2 g/l before, between and after passes. The suspension of lysed cells was divided into 300 ml portions, heated to 65°C for 1 hr., cooled down to 4°C, and centrifuged for 30 min. at 13,500 x g.

Following the centrifugation, NaCl and polyethyleneimine (PEI) (10% w/v, pH 7.0) were added to the heat-treated supernatant to a final concentration of 0.5 M and to 0.1%, respectively. The sample was mixed well and centrifuged at 13,500 x g for 1 hour.

The supernatant from the twice-centrifuged, heat-treated lysate was desalted by diluting with 10 liters of DE52 column buffer (20 mM Tris-HCl, pH 8.0; 0.5 mM EDTA; 7 mM βME) and concentrated to approximately 4 liters using an Amicon S10Y30 Spiral Ultrafiltration cartridge. The dilution/concentration step was repeated two times, with a final concentrated volume of about 4 liters.

The desalted sample was batch contacted with 400 g of equilibrated Whatman DE52 ion exchange resin (Maidstone, England). The suspension was collected on a sintered glass funnel and washed 3 times with 1 volume of DE52 column buffer. The resin was then resuspended in a minimal volume of buffer and poured into a column (4.5 x 50 cm), packed and washed with an additional volume of buffer. The column was eluted with a 0-0.5 M NaCl linear gradient (total gradient volume: 2000 ml). Twenty-five ml fractions were collected at a rate of about 5 ml/min. Peak fractions (fractions containing DNA polymerase activity) were determined by a modified DNA polymerase assay described by Kaledin et al., *Biokhimiya 45*:644-651 (1980), pooled and dialyzed in approximately twenty-five volumes of Affi-Gel Blue (AGB) column buffer (20 mM Tris-HCl, pH 7.5; 0.5 mM EDTA; 10 mM βME; 10 mM $MgCl_2$; 0.02% Brij 35).

The dialyzed DE52 peak fractions were applied to an AGB column (4.4 x 40 cm, 600 ml packed volume, MBR Blue, Molecular Biology Resources, Milwaukee WI), which was washed with 2 column volumes of AGB column buffer, and eluted with a 0-1.2 M NaCl linear gradient (total gradient volume: 2000 ml). To elute the exo⁻ fragment, a 0-1.5 M NaCl linear gradient was employed. Twenty-five ml fractions were collected at a

rate of 1-5 ml/min. The peak fractions were dialyzed as above in AGB buffer.

The dialyzed AGB peak fractions were applied to a Heparin Agarose column (4.4 x 16.5 cm, 250 ml packed volume (Bio-Rad Affigel Heparin or Heparin Agarose from Molecular Chimerics, Madison, WI)), which was washed with approximately 2 column volumes (until effluent is no longer colored, and column resin is white in appearance), and eluted with a 0.1-1.0 M NaCl linear gradient (total gradient volume: 1500 ml). To elute the exo fragment, a 0.15-1.0 M NaCl linear gradient was employed. Twenty-five ml fractions were collected at a rate of 1-5 ml/min. The peak fractions were dialyzed in HP Q Sepharose Column Buffer (20 mM Tris-HCl, pH 7.5; 0.5 mM EDTA; 7 mM βME; 0.1% Brij 35).

The dialyzed heparin agarose peak fractions were filtered through a 0.2 μm filter and applied at 4 ml/min. to the HP Q Sepharose column (Pharmacia, Uppsala, Sweden) on FPLC. The column was washed with several column volumes of buffer, and eluted with a 0-0.25 M NaCl linear gradient. Ten ml fractions were collected at 4 ml/minute. The peak fractions were dialyzed in HP S Column Buffer (20 mM Na-Citrate, pH 6.0; 1 mM EDTA; 7 mM βME; 0.1% Brij 35) or diluted in the same buffer, depending on the volume of the fraction pool.

The dialyzed (or diluted) HP Q peak fractions were filtered through a 0.2 μm filter and the HP S column (Pharmacia) was run as above, washing with HP S Column buffer and eluting with a 0-0.25 M NaCl gradient. Peak fractions were pooled and dialyzed against 4 liters of Final Storage Buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM DTT, 50% glycerol). The final product was diluted to a concentration of 5000 U/ml in the above buffer including 0.5 % Tween 20 (Sigma Chemical Co., St. Louis, MO) and 0.5 % Nonidet P40 (Fluka Biochemika, Buchs, Switzerland) as stabilizers and stored at -20°C. To purify the recombinant holoenzyme, the HP S column purification was unnecessary, and therefore was omitted.

Samples from the above-described preparations were electrophoresed using SDS-Page and visualized with silver staining. The rTfl holoenzyme and exo⁻ fragments appeared as single bands having apparent molecular weights of 88,000 and 63,000 kDa, respectively, each being greater than 95% pure. A quantitative analysis of the enzymes prepared using the above-described purification procedure is as shown in Table 6:

TABLE 6

Enzyme	Quantity of Cells	Specific Activity (Units/mg protein)	Yield (Units/g cells)
nTfl Holo (Example 1)	1200 g	50,000 U/mg	1,700 U/g
rTfl Holo	460 g	70,000 U/mg	4,300 U/g
rTfl exo⁻	787 g	192,000 U/mg	5,600 U/g

The biological activities of the recombinant enzymes purified by the above-described protocol were analyzed using the assays described in preceding Examples. In the endonuclease activity assay described in Example 9, five, ten, and twenty unit challenges resulted in less than 5% conversion of supercoiled pBR322 to the linear form. The results of other assays described in Example 9 are summarized in Table 7:

TABLE 7

ASSAY	rTfl holo	Tfl Exo⁻
ds DNase	0% slope/unit	0% slope/unit
ss DNase	0% slope/unit	0% slope/unit
3' Exonuclease	0% slope/unit	0.06% slope/unit
5' Exonuclease	0.48% slope/unit	0% slope/unit

Deposit of Biological Materials: The following plasmids have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville MD 20852 (USA) pursuant to the provisions of the Budapest Treaty:

Designation	Deposit Date	ATCC No.	Host Strain
pTFLRT4	May 26, 1994	69633	DH5αF'IQ
P21EHcM1.1	May 26, 1994	69632	DH5αF'

Availability of the deposited materials is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The present invention has been described with reference to specific examples and embodiments. However, this application is intended to cover those changes and substitutions which are apparent and may be made by those skilled in the art without departing from the spirit and scope of the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Molecular Biology Resources, Inc.
(B) STREET: 5520 W. Burleigh Street
(C) CITY: Milwaukee
(D) STATE: Wisconsin
(E) COUNTRY: United States of America
(F) POSTAL CODE: 53210

(ii) TITLE OF INVENTION: Biologically Active Fragments of Thermus Flavus DNA Polymerase

(iii) NUMBER OF SEQUENCES: 51

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
(B) STREET: 6300 Sears Tower, 233 South Wacker Drive
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: United States of America
(F) POSTAL CODE: 60606-6402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Gass, David A.
(B) REGISTRATION NUMBER: 38,153
(C) REFERENCE/DOCKET NUMBER: 28003/31716

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 312/474-6300
(B) TELEFAX: 312/474-0448
(C) TELEX: 25-3856

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3048 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 301..2805

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TACTTCGGCG GGGTGAAGCT CGGGGCCGGG GGGCTTGTGC GGGCCTACGG GGGGGTGGCG      60

GCGGAGGCCT TAAGCGGGCG CCCAAGGTCC CCTTGGTGGA GCGGGTGGGG CTCGCCTTCC     120

TCGTGCCCTT CGCCGAGGTG GGCCGGGTCT ACGCCCTCCT GGAGGCCCGC GCCCTGAAGG     180

CCGAGGAGAC CTACACCCCG GAGGGCGTGC GCTTCGCCCT CCTCCTCCCC AAGCCCGAGC     240

GGGAAGGTTT CCTCAGGGCG CTCCTGGACG CCACCCGGGG ACAGGTGGCC CTGGAGTAGC     300

ATG GAG GCG ATC GTT CCG CTC TTT GAA CCC AAA GGC CGG GTC CTC CTG         348
Met Glu Ala Ile Val Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
  1               5                  10                  15

GTG GAC GGC CAC CAC CTG GCC TAC CGC ACC TTC TTC GCC CTG AAG GGC         396
Val Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly
            20                  25                  30

CTC ACC ACG AGC CGG GGC GAA CCG GTG CAG GCG GTC TAC GGC TTC GCC         444
Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
        35                  40                  45

AAG AGC CTC CTC AAG GCC CTG AAG GAG GAC GGG TAC AAG GCC GTC TTC         492
Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Tyr Lys Ala Val Phe
    50                  55                  60

GTG GTC TTT GAC GCC AAG GCC CCC TCC TTC CGC CAC GAG GCC TAC GAG         540
Val Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu
65                  70                  75                  80

GCC TAC AAG GCG GGG AGG GCC CCG ACC CCC GAG GAC TTC CCC CGG CAG         588
Ala Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln
                85                  90                  95

CTC GCC CTC ATC AAG GAG CTG GTG GAC CTC CTG GGG TTT ACC CGC CTC         636
Leu Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Phe Thr Arg Leu
            100                 105                 110

GAG GTC CCC GGC TAC GAG GCG GAC GAC GTC CTC GCC ACC CTG GCC AAG         684
Glu Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys
        115                 120                 125

AAG GCG GAA AAG GAG GGG TAC GAG GTG CGC ATC CTC ACC GCC GAC CGC         732
Lys Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg
    130                 135                 140

GAC CTC TAC CAA CTC GTC TCC GAC CGC GTC GTC GTC CTC CAC CCC GAG         780
Asp Leu Tyr Gln Leu Val Ser Asp Arg Val Val Val Leu His Pro Glu
145                 150                 155                 160

GGC CAC CTC ATC ACC CCG GAG TGG CTT TGG GAG AAG TAC GGC CTC AAG         828
Gly His Leu Ile Thr Pro Glu Trp Leu Trp Glu Lys Tyr Gly Leu Lys
                165                 170                 175

CCG GAG CAG TGG GTG GAC TTC CGC GCC CTC GTG GGG GAC CCC TCC GAC         876
Pro Glu Gln Trp Val Asp Phe Arg Ala Leu Val Gly Asp Pro Ser Asp
            180                 185                 190
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AAC CTC CCC GGG GTC AAG GGC ATC GGG GAG AAG ACC GCC CTC AAG CTC        924
Asn Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu
        195                 200                 205

CTC AAG GAG TGG GGA AGC CTG GAA AAC CTC CTC AAG AAC CTG GAC CGG        972
Leu Lys Glu Trp Gly Ser Leu Glu Asn Leu Leu Lys Asn Leu Asp Arg
    210                 215                 220

GTA AAG CCA GAA AAC GTC CGG GAG AAG ATC AAG GCC CAC CTG GAA GAC       1020
Val Lys Pro Glu Asn Val Arg Glu Lys Ile Lys Ala His Leu Glu Asp
225                 230                 235                 240

CTC AGG CTT TCC TTG GAG CTC TCC CGG GTG CGC ACC GAC CTC CCC CTG       1068
Leu Arg Leu Ser Leu Glu Leu Ser Arg Val Arg Thr Asp Leu Pro Leu
                245                 250                 255

GAG GTG GAC CTC GCC CAG GGG CGG GAG CCC GAC CGG GAG GGG CTT AGG       1116
Glu Val Asp Leu Ala Gln Gly Arg Glu Pro Asp Arg Glu Gly Leu Arg
            260                 265                 270

GCC TTC CTG GAG AGG CTG GAG TTC GGC AGC CTC CTC CAC GAG TTC GGC       1164
Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly
        275                 280                 285

CTC CTG GAG GCC CCC GCC CCC CTG GAG GAG GCC CCC TGG CCC CCG CCG       1212
Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro
    290                 295                 300

GAA GGG GCC TTC GTG GGC TTC GTC CTC TCC CGC CCC GAG CCC ATG TGG       1260
Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp
305                 310                 315                 320

GCG GAG CTT AAA GCC CTG GCC GCC TGC AGG GAC GGC CGG GTG CAC CGG       1308
Ala Glu Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg Val His Arg
                325                 330                 335

GCA GCA GAC CCC TTG GCG GGG CTA AAG GAC CTC AAG GAG GTC CGG GGT       1356
Ala Ala Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly
            340                 345                 350

CTC CTC GCC AAG GAC CTC GCC GTC TTG GCC TCG AGG GAG GGG CTA GAC       1404
Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu Gly Leu Asp
        355                 360                 365

CTC GTG CCC GGG GAC GAC CCC ATG CTC CTC GCC TAC CTC CTG GAC CCC       1452
Leu Val Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro
    370                 375                 380

TCC AAC ACC ACC CCC GAG GGG GTG GCG CGG CGC TAC GGG GGG GAG TGG       1500
Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp
385                 390                 395                 400

ACG GAG GAC GCC GCC CAC CGG GCC CTC CTC TCG GAG AGG CTC CAT CGG       1548
Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg Leu His Arg
                405                 410                 415

AAC CTC CTT AAG CGC CTC GAG GGG GAG GAG AAG CTC CTT TGG CTC TAC       1596
Asn Leu Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu Trp Leu Tyr
            420                 425                 430
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CAC GAG GTG GAA AAG CCC CTC TCC CGG GTC CTG GCC CAC ATG GAG GCC          1644
His Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala
        435                 440                 445

ACC GGG GTA CGG CTG GAC GTG GCC TAC CTG CAG GCC CTT TCC CTG GAG          1692
Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu
    450                 455                 460

CTT GCG GAG GAG ATC CGC CGC CTC GAG GAG GAG GTC TTC CGC TTG GCG          1740
Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala
465                 470                 475                 480

GGC CAC CCC TTC AAC CTC AAC TCC CGG GAC CAG CTG GAA AGG GTG CTC          1788
Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu
                485                 490                 495

TTT GAC GAG CTT AGG CTT CCC GCC TTG GGG AAG ACG CAA AAG ACG GGC          1836
Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly
            500                 505                 510

AAG CGC TCC ACC AGC GCC GCG GTG CTG GAG GCC CTA CGG GAG GCC CAC          1884
Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His
        515                 520                 525

CCC ATC GTG GAG AAG ATC CTC CAG CAC CGG GAG CTC ACC AAG CTC AAG          1932
Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys
    530                 535                 540

AAC ACC TAC GTG GAC CCC CTC CCA AGC CTC GTC CAC CCG AGG ACG GGC          1980
Asn Thr Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro Arg Thr Gly
545                 550                 555                 560

CGC CTC CAC ACC CGC TTC AAC CAG ACG GCC ACG GCC ACG GGG AGG CTT          2028
Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu
                565                 570                 575

AGT AGC TCC GAC CCC AAC CTG CAG AAC ATC CCC GTC CGC ACC CCC TTG          2076
Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu
            580                 585                 590

GGC CAG AGG ATC CGC CGG GCC TTC GTG GCC GAG GCG GGA TGG GCG TTG          2124
Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu
        595                 600                 605

GTG GCC CTG GAC TAT AGC CAG ATA GAG CTC CGC GTC CTC GCC CAC CTC          2172
Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu
    610                 615                 620

TCC GGG GAC GAG AAC CTG ATC AGG GTC TTC CAG GAG GGG AAG GAC ATC          2220
Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile
625                 630                 635                 640

CAC ACC CAG ACC GCA AGC TGG ATG TTC GGC GTC CCC CCG GAG GCC GTG          2268
His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro Glu Ala Val
                645                 650                 655

GAC CCC CTG ATG CGC CGG GCG GCC AAG ACG GTG AAC TTC GGC GTC CTC          2316
Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu
            660                 665                 670
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TAC GGC ATG TCC GCC CAT AGG CTC TCC CAG GAG CTT GCC ATC CCC TAC      2364
Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr
        675                 680                 685

GAG GAG GCG GTG GCC TTT ATA GAG CGC TAC TTC CAA AGC TTC CCC AAG      2412
Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys
    690                 695                 700

GTG CGG GCC TGG ATA GAA AAG ACC CTG GAG GAG GGG AGG AAG CGG GGC      2460
Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly
705                 710                 715                 720

TAC GTG GAA ACC CTC TTC GGA AGA AGG CGC TAC GTG CCC GAC CTC AAC      2508
Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn
                725                 730                 735

GCC CGG GTG AAG AGC GTC AGG GAG GCC GCG GAG CGC ATG GCC TTC AAC      2556
Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn
            740                 745                 750

ATG CCC GTC CAG GGC ACC GCC GCC GAC CTC ATG AAG CTC GCC ATG GTG      2604
Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val
        755                 760                 765

AAG CTC TTC CCC CGC CTC CGG GAG ATG GGG GCC CGC ATG CTC CTC CAG      2652
Lys Leu Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln
    770                 775                 780

GTC CAC GAC GAG CTC CTC CTG GAG GCC CCC CAA GCG CGG GCC GAG GAG      2700
Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu
785                 790                 795                 800

GTG GCG GCT TTG GCC AAG GAG GCC ATG GAG AAG GCC TAT CCC CTC GCC      2748
Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala
                805                 810                 815

GTG CCC CTG GAG GTG GAG GTG GGG ATG GGG GAG GAC TGG CTT TCC GCC      2796
Val Pro Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala
            820                 825                 830

AAG GGT TAGGGGGGCC CTGCCGTTTA GAGGAAGTTC AAGGGGTTGT CCCTCAGAAA      2852
Lys Gly

CGCCTCCAGG GGAACGCCCT CTGCGGCTAC CAGGAGGCCT TTAGCCCCAA AGGTGCGGGT   2912

GAAGGCTTCC AGGCCCTGGG TTCTTTTAAA GGGGGCGCTT TTGACCTCGA GGGCCAGGAG   2972

GCGCTTTCCC TTTTGAAGGA CAAAGTCACT TCCTGGTCCC TTTCCCGCCA GTAGTACACC   3032

TCAAACCCCC CCTGGT                                                   3048
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 834 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Ala Ile Val Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
 1               5                  10                  15

Val Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly
            20                  25                  30

Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
        35                  40                  45

Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Tyr Lys Ala Val Phe
    50                  55                  60

Val Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu
65                  70                  75                  80

Ala Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln
                85                  90                  95

Leu Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Phe Thr Arg Leu
            100                 105                 110

Glu Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys
        115                 120                 125

Lys Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg
    130                 135                 140

Asp Leu Tyr Gln Leu Val Ser Asp Arg Val Val Val Leu His Pro Glu
145                 150                 155                 160

Gly His Leu Ile Thr Pro Glu Trp Leu Trp Glu Lys Tyr Gly Leu Lys
                165                 170                 175

Pro Glu Gln Trp Val Asp Phe Arg Ala Leu Val Gly Asp Pro Ser Asp
            180                 185                 190

Asn Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu
        195                 200                 205

Leu Lys Glu Trp Gly Ser Leu Glu Asn Leu Leu Lys Asn Leu Asp Arg
    210                 215                 220

Val Lys Pro Glu Asn Val Arg Glu Lys Ile Lys Ala His Leu Glu Asp
225                 230                 235                 240

Leu Arg Leu Ser Leu Glu Leu Ser Arg Val Arg Thr Asp Leu Pro Leu
                245                 250                 255

Glu Val Asp Leu Ala Gln Gly Arg Glu Pro Asp Arg Glu Gly Leu Arg
            260                 265                 270

Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly
        275                 280                 285

Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro
    290                 295                 300

Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp
305                 310                 315                 320

Ala Glu Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg Val His Arg
                325                 330                 335
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Ala Ala Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly
            340                 345                 350

Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu Gly Leu Asp
        355                 360                 365

Leu Val Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro
    370                 375                 380

Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp
385                 390                 395                 400

Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg Leu His Arg
                405                 410                 415

Asn Leu Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu Trp Leu Tyr
            420                 425                 430

His Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala
        435                 440                 445

Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu
    450                 455                 460

Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala
465                 470                 475                 480

Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu
                485                 490                 495

Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly
            500                 505                 510

Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His
        515                 520                 525

Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys
    530                 535                 540

Asn Thr Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro Arg Thr Gly
545                 550                 555                 560

Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu
                565                 570                 575

Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu
            580                 585                 590

Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu
        595                 600                 605

Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu
    610                 615                 620

Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile
625                 630                 635                 640

His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro Glu Ala Val
                645                 650                 655

Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu
            660                 665                 670
```

```
Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr
        675                 680                 685

Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys
    690                 695                 700

Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly
705                 710                 715                 720

Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn
                725                 730                 735

Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn
            740                 745                 750

Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val
        755                 760                 765

Lys Leu Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln
    770                 775                 780

Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu
785                 790                 795                 800

Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala
                805                 810                 815

Val Pro Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala
            820                 825                 830

Lys Gly
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1794 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1794

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG GAA GAC CTC AGG CTT TCC TTG GAG CTC TCC CGG GTG CGC ACC GAC        48
Met Glu Asp Leu Arg Leu Ser Leu Glu Leu Ser Arg Val Arg Thr Asp
  1               5                  10                  15

CTC CCC CTG GAG GTG GAC CTC GCC CAG GGG CGG GAG CCC GAC CGG GAG        96
Leu Pro Leu Glu Val Asp Leu Ala Gln Gly Arg Glu Pro Asp Arg Glu
            20                  25                  30

GGG CTT AGG GCC TTC CTG GAG AGG CTG GAG TTC GGC AGC CTC CTC CAC       144
Gly Leu Arg Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His
        35                  40                  45
```

```
GAG TTC GGC CTC CTG GAG GCC CCC GCC CCC CTG GAG GAG GCC CCC TGG          192
Glu Phe Gly Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp
     50                  55                  60

CCC CCG CCG GAA GGG GCC TTC GTG GGC TTC GTC CTC TCC CGC CCC GAG          240
Pro Pro Pro Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu
 65                  70                  75                  80

CCC ATG TGG GCG GAG CTT AAA GCC CTG GCC GCC TGC AGG GAC GGC CGG          288
Pro Met Trp Ala Glu Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg
                 85                  90                  95

GTG CAC CGG GCA GCA GAC CCC TTG GCG GGG CTA AAG GAC CTC AAG GAG          336
Val His Arg Ala Ala Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu
            100                 105                 110

GTC CGG GGT CTC CTC GCC AAG GAC CTC GCC GTC TTG GCC TCG AGG GAG          384
Val Arg Gly Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu
        115                 120                 125

GGG CTA GAC CTC GTG CCC GGG GAC GAC CCC ATG CTC CTC GCC TAC CTC          432
Gly Leu Asp Leu Val Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu
    130                 135                 140

CTG GAC CCC TCC AAC ACC ACC CCC GAG GGG GTG GCG CGG CGC TAC GGG          480
Leu Asp Pro Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly
145                 150                 155                 160

GGG GAG TGG ACG GAG GAC GCC GCC CAC CGG GCC CTC CTC TCG GAG AGG          528
Gly Glu Trp Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg
                165                 170                 175

CTC CAT CGG AAC CTC CTT AAG CGC CTC GAG GGG GAG GAG AAG CTC CTT          576
Leu His Arg Asn Leu Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu
            180                 185                 190

TGG CTC TAC CAC GAG GTG GAA AAG CCC CTC TCC CGG GTC CTG GCC CAC          624
Trp Leu Tyr His Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His
        195                 200                 205

ATG GAG GCC ACC GGG GTA CGG CTG GAC GTG GCC TAC CTG CAG GCC CTT          672
Met Glu Ala Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu
    210                 215                 220

TCC CTG GAG CTT GCG GAG GAG ATC CGC CGC CTC GAG GAG GAG GTC TTC          720
Ser Leu Glu Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe
225                 230                 235                 240

CGC TTG GCG GGC CAC CCC TTC AAC CTC AAC TCC CGG GAC CAG CTG GAA          768
Arg Leu Ala Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu
                245                 250                 255

AGG GTG CTC TTT GAC GAG CTT AGG CTT CCC GCC TTG GGG AAG ACG CAA          816
Arg Val Leu Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln
            260                 265                 270

AAG ACG GGC AAG CGC TCC ACC AGC GCC GCG GTG CTG GAG GCC CTA CGG          864
Lys Thr Gly Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg
        275                 280                 285
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```
GAG GCC CAC CCC ATC GTG GAG AAG ATC CTC CAG CAC CGG GAG CTC ACC          912
Glu Ala His Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr
    290                 295                 300

AAG CTC AAG AAC ACC TAC GTG GAC CCC CTC CCA AGC CTC GTC CAC CCG          960
Lys Leu Lys Asn Thr Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro
305                 310                 315                 320

AGG ACG GGC CGC CTC CAC ACC CGC TTC AAC CAG ACG GCC ACG GCC ACG         1008
Arg Thr Gly Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr
                325                 330                 335

GGG AGG CTT AGT AGC TCC GAC CCC AAC CTG CAG AAC ATC CCC GTC CGC         1056
Gly Arg Leu Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg
            340                 345                 350

ACC CCC TTG GGC CAG AGG ATC CGC CGG GCC TTC GTG GCC GAG GCG GGA         1104
Thr Pro Leu Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly
        355                 360                 365

TGG GCG TTG GTG GCC CTG GAC TAT AGC CAG ATA GAG CTC CGC GTC CTC         1152
Trp Ala Leu Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu
    370                 375                 380

GCC CAC CTC TCC GGG GAC GAG AAC CTG ATC AGG GTC TTC CAG GAG GGG         1200
Ala His Leu Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly
385                 390                 395                 400

AAG GAC ATC CAC ACC CAG ACC GCA AGC TGG ATG TTC GGC GTC CCC CCG         1248
Lys Asp Ile His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro
                405                 410                 415

GAG GCC GTG GAC CCC CTG ATG CGC CGG GCG GCC AAG ACG GTG AAC TTC         1296
Glu Ala Val Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe
            420                 425                 430

GGC GTC CTC TAC GGC ATG TCC GCC CAT AGG CTC TCC CAG GAG CTT GCC         1344
Gly Val Leu Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala
        435                 440                 445

ATC CCC TAC GAG GAG GCG GTG GCC TTT ATA GAG CGC TAC TTC CAA AGC         1392
Ile Pro Tyr Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser
    450                 455                 460

TTC CCC AAG GTG CGG GCC TGG ATA GAA AAG ACC CTG GAG GAG GGG AGG         1440
Phe Pro Lys Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg
465                 470                 475                 480

AAG CGG GGC TAC GTG GAA ACC CTC TTC GGA AGA AGG CGC TAC GTG CCC         1488
Lys Arg Gly Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro
                485                 490                 495

GAC CTC AAC GCC CGG GTG AAG AGC GTC AGG GAG GCC GCG GAG CGC ATG         1536
Asp Leu Asn Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met
            500                 505                 510

GCC TTC AAC ATG CCC GTC CAG GGC ACC GCC GCC GAC CTC ATG AAG CTC         1584
Ala Phe Asn Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu
        515                 520                 525
```

```
GCC ATG GTG AAG CTC TTC CCC CGC CTC CGG GAG ATG GGG GCC CGC ATG      1632
Ala Met Val Lys Leu Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met
    530                 535                 540

CTC CTC CAG GTC CAC GAC GAG CTC CTC CTG GAG GCC CCC CAA GCG CGG      1680
Leu Leu Gln Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg
545                 550                 555                 560

GCC GAG GAG GTG GCG GCT TTG GCC AAG GAG GCC ATG GAG AAG GCC TAT      1728
Ala Glu Glu Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr
                565                 570                 575

CCC CTC GCC GTG CCC CTG GAG GTG GAG GTG GGG ATG GGG GAG GAC TGG      1776
Pro Leu Ala Val Pro Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp
            580                 585                 590

CTT TCC GCC AAG GGT TAG                                              1794
Leu Ser Ala Lys Gly
        595
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 597 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Glu Asp Leu Arg Leu Ser Leu Glu Leu Ser Arg Val Arg Thr Asp
 1               5                  10                  15

Leu Pro Leu Glu Val Asp Leu Ala Gln Gly Arg Glu Pro Asp Arg Glu
            20                  25                  30

Gly Leu Arg Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His
        35                  40                  45

Glu Phe Gly Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp
    50                  55                  60

Pro Pro Pro Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu
65                  70                  75                  80

Pro Met Trp Ala Glu Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg
                85                  90                  95

Val His Arg Ala Ala Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu
            100                 105                 110

Val Arg Gly Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu
        115                 120                 125

Gly Leu Asp Leu Val Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu
    130                 135                 140

Leu Asp Pro Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly
145                 150                 155                 160
```

```
Gly Glu Trp Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg
                165                 170                 175

Leu His Arg Asn Leu Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu
            180                 185                 190

Trp Leu Tyr His Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His
        195                 200                 205

Met Glu Ala Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu
    210                 215                 220

Ser Leu Glu Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe
225                 230                 235                 240

Arg Leu Ala Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu
                245                 250                 255

Arg Val Leu Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln
            260                 265                 270

Lys Thr Gly Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg
        275                 280                 285

Glu Ala His Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr
    290                 295                 300

Lys Leu Lys Asn Thr Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro
305                 310                 315                 320

Arg Thr Gly Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr
                325                 330                 335

Gly Arg Leu Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg
            340                 345                 350

Thr Pro Leu Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly
        355                 360                 365

Trp Ala Leu Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu
    370                 375                 380

Ala His Leu Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly
385                 390                 395                 400

Lys Asp Ile His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro
                405                 410                 415

Glu Ala Val Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe
            420                 425                 430

Gly Val Leu Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala
        435                 440                 445

Ile Pro Tyr Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser
    450                 455                 460

Phe Pro Lys Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg
465                 470                 475                 480

Lys Arg Gly Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro
                485                 490                 495
```

```
Asp Leu Asn Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met
            500                 505                 510

Ala Phe Asn Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu
        515                 520                 525

Ala Met Val Lys Leu Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met
    530                 535                 540

Leu Leu Gln Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg
545                 550                 555                 560

Ala Glu Glu Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr
                565                 570                 575

Pro Leu Ala Val Pro Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp
            580                 585                 590

Leu Ser Ala Lys Gly
        595
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 560 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly Leu Leu
  1               5                  10                  15

Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly
            20                  25                  30

Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp Ala Glu
        35                  40                  45

Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg Val His Arg Ala Ala
    50                  55                  60

Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly Leu Leu
65                  70                  75                  80

Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu Gly Leu Asp Leu Val
                85                  90                  95

Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn
            100                 105                 110

Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu
        115                 120                 125

Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg Leu His Arg Asn Leu
    130                 135                 140
```

```
Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu Trp Leu Tyr His Glu
145                 150                 155                 160

Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala Thr Gly
                165                 170                 175

Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu Leu Ala
            180                 185                 190

Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala Gly His
        195                 200                 205

Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu Phe Asp
    210                 215                 220

Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly Lys Arg
225                 230                 235                 240

Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His Pro Ile
                245                 250                 255

Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys Asn Thr
            260                 265                 270

Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro Arg Thr Gly Arg Leu
        275                 280                 285

His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser
    290                 295                 300

Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu Gly Gln
305                 310                 315                 320

Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu Val Ala
                325                 330                 335

Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly
            340                 345                 350

Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile His Thr
        355                 360                 365

Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro Glu Ala Val Asp Pro
    370                 375                 380

Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu Tyr Gly
385                 390                 395                 400

Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
                405                 410                 415

Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg
            420                 425                 430

Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly Tyr Val
        435                 440                 445

Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn Ala Arg
    450                 455                 460

Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro
465                 470                 475                 480
```

Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Lys Leu
485 490 495

Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln Val His
500 505 510

Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu Val Ala
515 520 525

Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala Val Pro
530 535 540

Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala Lys Gly
545 550 555 560

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCAGGGTT TTCCCAGTCA CGAC 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGCGGATAAC AATTTCACAC AGGA 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTAAGTAGCT CCGATCCCAA C 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCACTCCTT GGCGGAGAGC CAGTC 25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATTTAGCACA TATGGCGATG CTTCCC 26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTTTCCAGCT CCGACCCCAA C 21

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCTACTCCTT GGCGGAGAGC CAGTC 25

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGGATGTCCC TCCCCTCCTG AAAGA 25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCCTTTCCCG GAAGCTTTCC CAGGTGCA 28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TGCACCTGGG AAAGCTTCCG GGAAAGGG 28

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCTGCAGTAC CGGGAGCTCA CCAAGCTCAA 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTGAGCTTGG TGAGCTCCCG GTACTGCAGG 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGGACTATAG CCAGATAGAG CT 22

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAGCGAAGAC CTCCTCCTCG A 21

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGTTCGGCAG CCTCCTCCAC GA 22

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCCAAGGAAA GCCTGAGGTC TT 22

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AAGCTCGCCA TGGTGAAGCT CTT 23

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCGGAGACGA GTTGGTAGAG GT 22

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ACCTCTACCA ACTCGTCTCC GA 22

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AGAGGACGAA GCCCACGAA 19

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGGAGGTAGG CGAGGAGCAT 20

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATGCTCCTCG CCTACCTCCT 20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TCGAGGAGGA GGTCTTCGCT T 21

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AGCTCTATCT GGCTATAGTC CA 22

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATAGGCTCTC CCAGGAGCTT 20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AAGAGCTTCA CCATGGCGAG CTT 23

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TTCCCCTGGA GGCGTTTCTG A 21

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AAAGACCACG AAGACGGCCT T 21

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AAGGCCGTCT TCGTGGTCTT T 21

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

AAGGAGTGGG GAAGCCTGGA A 21

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TTCCAGGCTT CCCCACTCCT T 21

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TTCTTCCGAA GAGGGTTTCC A 21

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GCGTCCAGGA GCGCCCTGAG GA 22

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCTCAGGGCG CTCCTGGACG CCA 23

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TTCGTCCTCT CCCGCCCCGA 20

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCAACCTGCA GAACATCCCC GT 22

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GGTGTGGATG TCCTTCCCCT 20

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCCTGCCGTT TAGAGGAAGT TCAAG 25

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CTTGAACTTC CTCTAAACGG CAGGG 25

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

ACCCGGCCTT TGGGTTCAAA GA 22

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TCTTTGAACC CAAAGGCCGG GT 22

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TTCCCGTGCT CCTTCCGCTC 20

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CTCGCCTTCC TCGTGCCCTT 20

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GCTTCCGGCT CGTATGTTGT GTG 23

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GGAAAGCCTG AGGTCTTCCA TAGCTGTTTC CTGTGTGAAA TTGTTATCCG CTCACAATTC 60

CACACAACAT 70

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

ACCCGGCCTT TGGGTTCAAA GAGCGGAACG ATCGCCTCCA TAGCTGTTTC CTGTGTGAAA 60

TTGTTATCCG CTCACAATTC C 81

What is Claimed is:

1. A purified DNA in accordance with SEQ ID NO: 3, said DNA encoding for a member selected from the group consisting of a polypeptide in accordance with SEQ ID NO: 5, and fragments thereof having polymerase activity.

2. The DNA of claim 1 consisting of nucleotides 112 to 1791 of SEQ ID NO: 3.

3. The DNA of claim 1 consisting of nucleotides 1 to 1791 of SEQ. ID NO: 3.

4. A vector wherein the DNA of SEQ. ID NO: 3 is operably linked to a promoter.

5. Plasmid p21EHcM1.1, having ATCC Accession No. 69632.

6. A host cell transformed with a DNA selected from the group consisting of the DNAs of claims 1, 2, and 3.

7. The host cell of claim 6, wherein said host cell is capable of expressing a thermostable polypeptide encoded by said DNA, said polypeptide having DNA polymerase activity.

8. The host cell of claim 7, wherein said host cell is a prokaryotic cell.

9. The host cell of claim 8, wherein said host cell is an *E. coli* cell.

10. An expression vector operably linked to nucleotides 112 to 1791 of SEQ ID NO: 3, said nucleotides encoding a polypeptide having thermostable DNA polymerase activity.

11. The expression vector of claim 10 having at least one insert consisting essentially of nucleotides 112 to 1791 of SEQ ID NO:3.

12. A purified fragment of *Thermus flavus* DNA polymerase I protein in accordance with SEQ ID NO: 5, said fragment having thermostable DNA polymerase activity.

13. A fragment of *Thermus flavus* DNA polymerase I having thermostable DNA polyerase activity and consisting of amino acids 2 to 560 of SEQ. ID NO: 5.

14. A purified fragment of *Thermus flavus* DNA polymerase I protein encoded by the insert of plasmid p21EHcM1.1, having ATCC Accession no. 69632.

15. The purified fragment of claim 14 wherein the fragment has a DNA polymerase activity between 60,000 U/mg protein and 600,000 U/mg protein.

16. A thermostable polypeptide having DNA polymerase activity, said polypeptide consisting essentially of the amino acid sequence of SEQ ID NO: 5.

17. A method for purifying a thermostable polypeptide having DNA polymerase activity comprising the steps of:

transforming a host cell with a DNA to create a transformed host cell, said DNA encoding for a thermostable polypeptide having DNA polymerase activity and being selected from the group consisting of the DNAs of claims 1, 2, and 3;

cultivating said transformed host cell under conditions to promote expression of a thermostable polypeptide encoded by said DNA; and

purifying said thermostable polypeptide with a monoclonal antibody that is immunologically cross-reactive with said thermostable polypeptide.

18. The method of claim 17 wherein the host cell is transformed with the DNA of claim 3.

19. The method of claim 17 wherein said immunologically cross-reactive monoclonal antibody has specificity for a *Thermus aquaticus* DNA polymerase.

20. A method of purifying a thermostable polypeptide having DNA polymerase activity comprising the steps of:

a) expressing said thermostable polypeptide in a host cell, said polypeptide having an amino acid sequence encoded by a DNA selected from the group consisting of the DNAs of claims 1, 2, and 3;

b) lysing the cell to create a suspension containing said thermostable polypeptide and host cell proteins and cell debris;

c) contacting a soluble portion of said suspension with an antibody that is immunologically cross-reactive with said thermostable polypeptide and under conditions wherein the antibody binds to said thermostable polypeptide to form an antibody-polypeptide complex;

d) isolating the antibody-polypeptide complex; and

e) separating said thermostable polypeptide from said isolated antibody-polypeptide complex to provide a purified thermostable polypeptide.

21. The method of claim 20 further comprising between steps (b) and (c) the steps of:

heating said suspension to denature host cell proteins; and

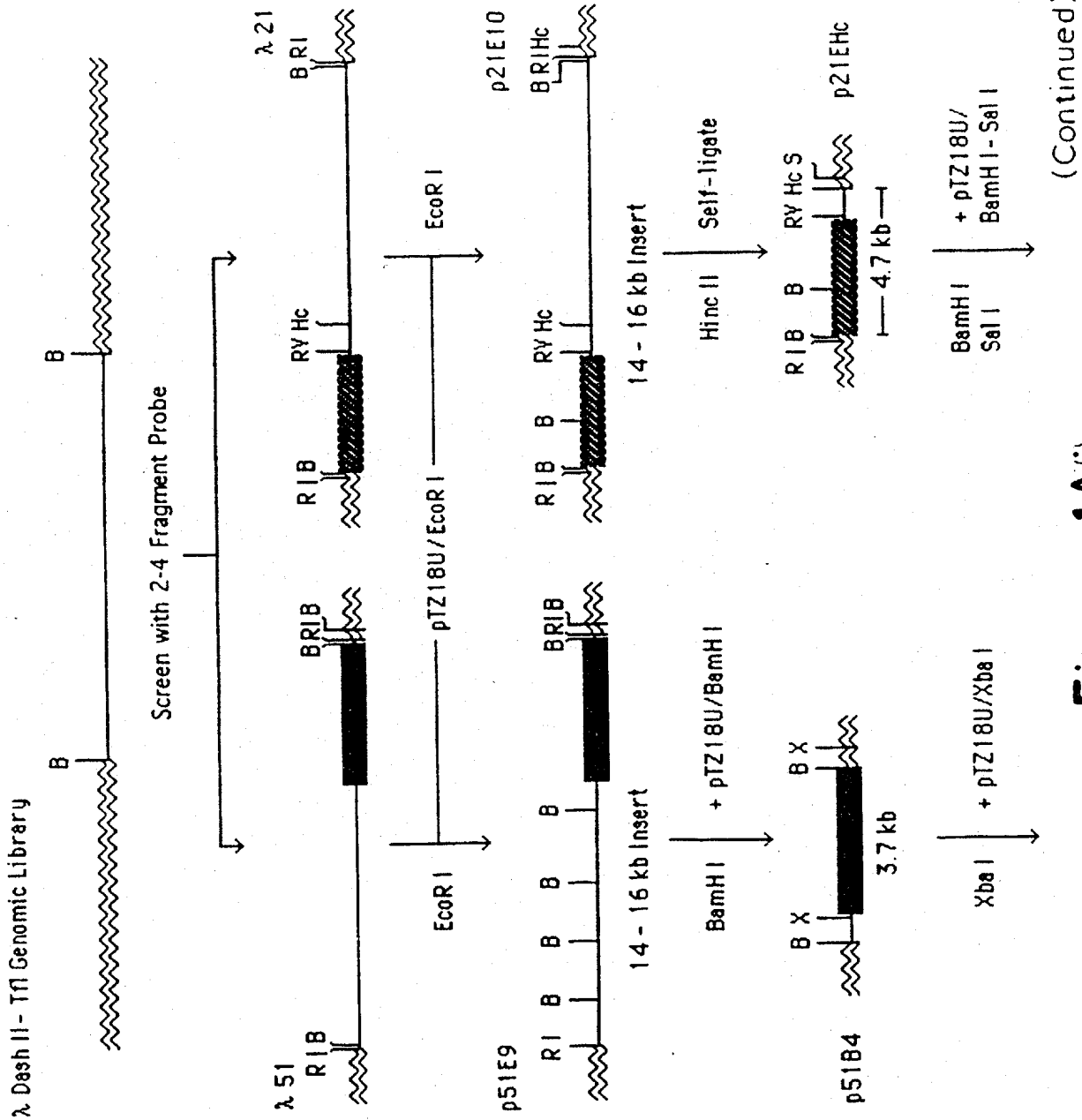
centrifuging said suspension to remove said cell debris and denatured host cell proteins.

22. The method of claim 20 or 21 wherein said immunologically cross-reactive antibody is a monoclonal antibody.

23. The method of claim 22 wherein said immunologically cross-reactive monoclonal antibody is specific for *Thermus aquaticus* DNA polymerase I.

24. The method of claim 22 wherein the purified thermostable polypeptide has a DNA polymerase activity between 79,500 U/mg protein and 600,000 U/mg protein.

25. The method of claim 22 wherein the purified thermostable polypeptide has a DNA polymerase activity between 217,600 U/mg protein and 600,000 U/mg protein.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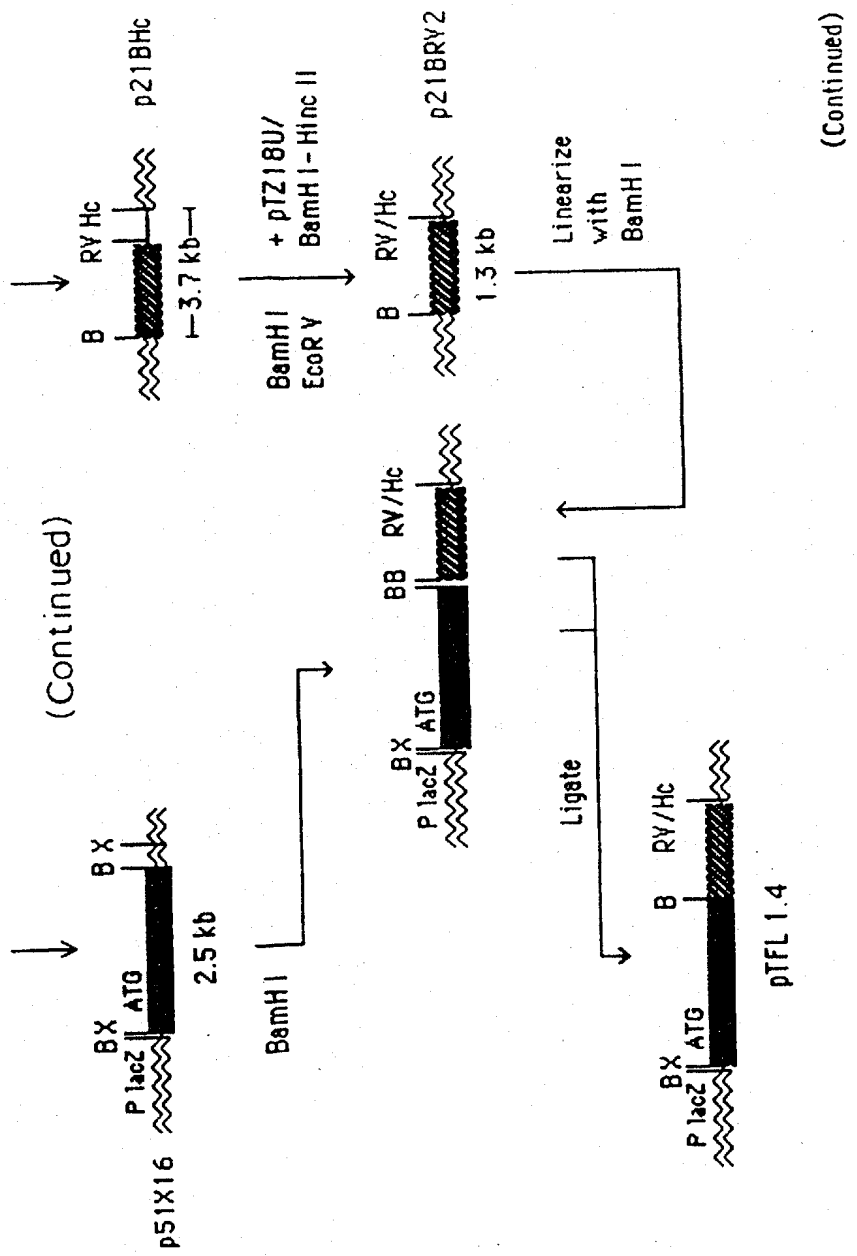
B
B
Screen with 2-4 Fragment Probe
λ 51
R I B
B R I B
λ 21
B R I
RV Hc
R I B
pTZ18U/EcoR I
EcoR I
EcoR I
p51E9
R I B B B B
B R I B
14 - 16 kb Insert
+ pTZ18U/BamH I
BamH I
p21E10
B R I Hc
RV Hc
R I B B
14 - 16 kb Insert
Hinc II
Self-ligate
p5184
B X
B X
3.7 kb
Xba I
+ pTZ18U/Xba I
p21EHc
RV Hc S
R I B B
4.7 kb
BamH I
Sal I
+ pTZ18U/
BamH I - Sal I
(Continued)

Figure 1A(i)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2.5 kb
BamH I
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ATG
B X
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BamH I - Hinc II
BamH I
EcoR V
B RV Hc
p21BHc
3.7 kb
B RV/Hc
p21BRV2
1.3 kb
Linearize
with
BamH I
Ligate
P lacZ
ATG
B X
B RV/Hc
pTFL 1.4
(Continued)

Figure 1A(ii)

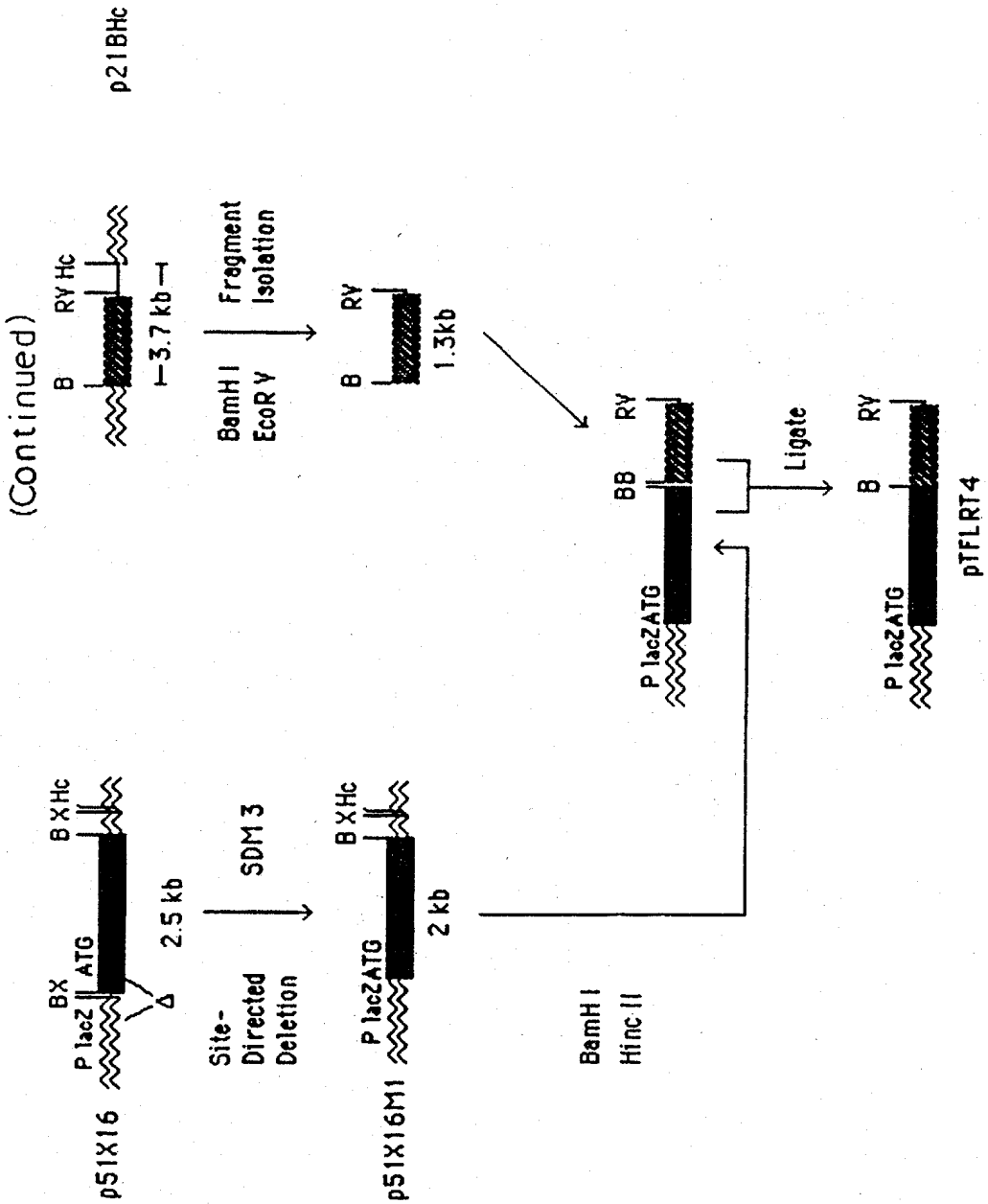


Figure 1A(iii)

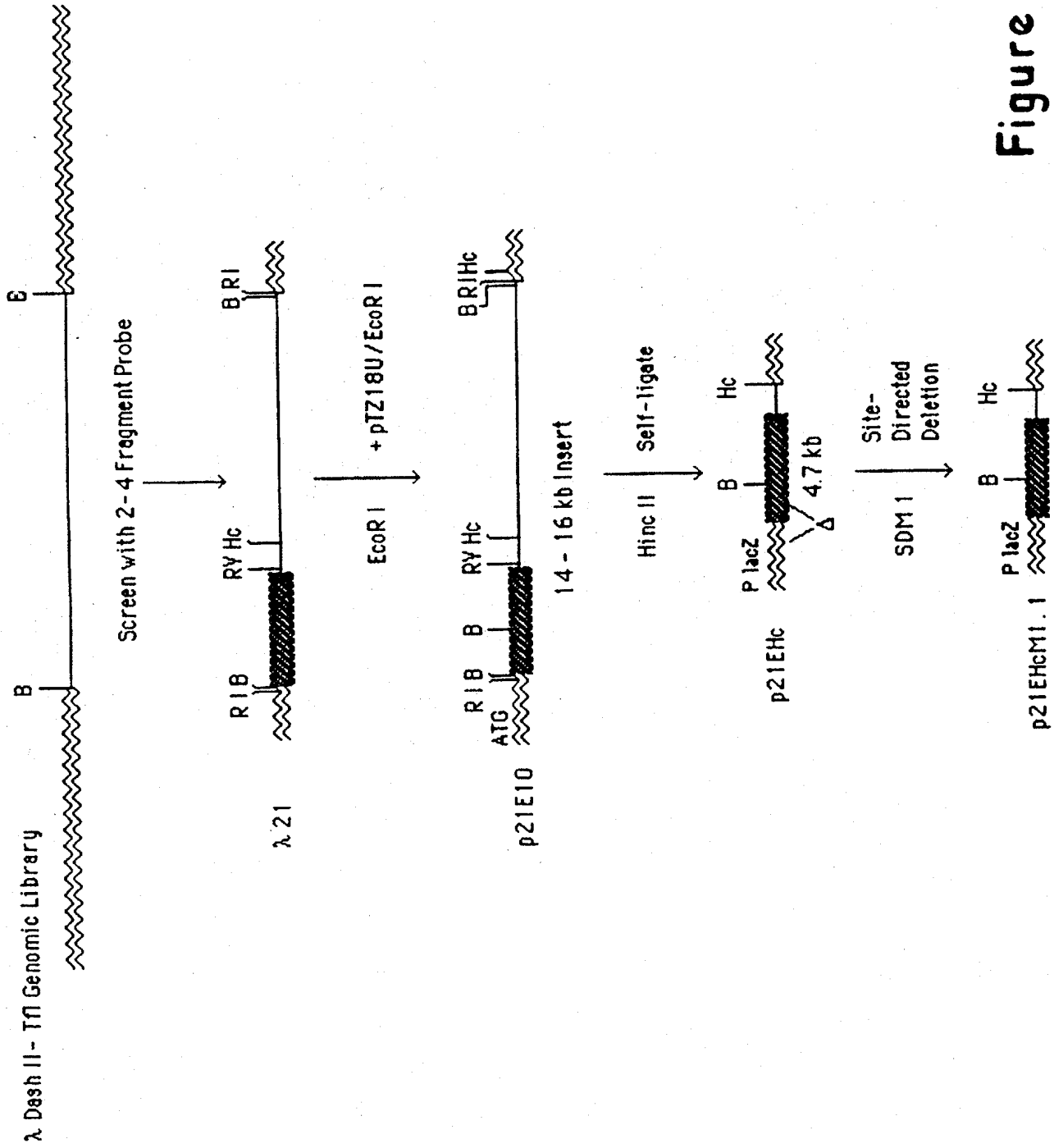

λ Dash II - Tfl Genomic Library
B
E
Screen with 2-4 Fragment Probe
λ 21
R I B
RV Hc
B R I
EcoR I
+ pTZ18U/EcoR I
p21E10
R I B
ATG
B
RV Hc
B R I Hc
14 - 16 kb Insert
Hinc II
Self-ligate
p21EHc
P lacZ
B
Hc
4.7 kb
Δ
SDM 1
Site-
Directed
Deletion
p21EHcM1.1
P lacZ
B
Hc

Figure 1B

```
TACTTCGGCGGGGTGAAGCTCGGGGCCGGGGGGCTTGTGCGGGCCTACGGGGGGGTGGCGGCGGAGGCCTTAAGCGGGCGCCCAAGGTCCCCTTGGTGGAGCGGGTGGGGCTCGCCTTCC   120

TCGTGCCCTTCGCCGAGGTGGGCCGGGTCTACGCCCTCCTGGAGGCCCGCGCCCTGAAGGCCGAGGAGACCTACACCCCGGAGGGCGTGCGCTTCGCCCTCCTCCTCCCCAAGCCCGAGC   240

GGGAAGGTTTCCTCAGGGCGCTCCTGGACGCCACCCGGGGACAGGTGGCCCTGGAGTAGCATG GAG GCG ATC GTT CCG CTC TTT GAA CCC AAA GGC CGG GTC CTC   345
                                                                  M  E  A  I  V  P  L  F  E  P  K  G  R  V  L    15

CTG GTG GAC GGC CAC CAC CTG GCC TAC CGC ACC TTC TTC GCC CTG AAG GGC CTC ACC ACG AGC CGG GGC GAA CCG GTG CAG GCG GTC TAC   435
 L  V  D  G  H  H  L  A  Y  R  T  F  F  A  L  K  G  L  T  T  S  R  G  E  P  V  Q  A  V  Y    45

GGC TTC GCC AAG AGC CTC CTC AAG GCC CTG AAG GAG GAC GGG TAC AAG GCC GTC TTC GTG GTC TTT GAC GCC AAG GCC CCC TCC TTC CGC   525
 G  F  A  K  S  L  L  K  A  L  K  E  D  G  Y  K  A  V  F  V  V  F  D  A  K  A  P  S  F  R    75

CAC GAG GCC TAC GAG GCC TAC AAG GCG GGG AGG GCC CCG ACC CCC GAG GAC TTC CCC CGG CAG CTC GCC CTC ATC AAG GAG CTG GTG GAC   615
 H  E  A  Y  E  A  Y  K  A  G  R  A  P  T  P  E  D  F  P  R  Q  L  A  L  I  K  E  L  V  D   105

CTC CTG GGG TTT ACC CGC CTC GAG GTC CCC GGC TAC GAG GCG GAC GAC GTC CTC GCC ACC CTG GCC AAG AAG GCG GAA AAG GAG GGG TAC   705
 L  L  G  F  T  R  L  E  V  P  G  Y  E  A  D  D  V  L  A  T  L  A  K  K  A  E  K  E  G  Y   135

GAG GTG CGC ATC CTC ACC GCC GAC CGC GAC CTC TAC CAA CTC GTC TCC GAC CGC GTC GTC GTC CTC CAC CCC GAG GGC CAC CTC ATC ACC   795
 E  V  R  I  L  T  A  D  R  D  L  Y  Q  L  V  S  D  R  V  V  V  L  H  P  E  G  H  L  I  T   165

CCG GAG TGG CTT TGG GAG AAG TAC GGC CTC AAG CCG GAG CAG TGG GTG GAC TTC CGC GCC CTC GTG GGG GAC CCC TCC GAC AAC CTC CCC   885
 P  E  W  L  W  E  K  Y  G  L  K  P  E  Q  W  V  D  F  R  A  L  V  G  D  P  S  D  N  L  P   195

GGG GTC AAG GGC ATC GGG GAG AAG ACC GCC CTC AAG CTC CTC AAG GAG TGG GGA AGC CTG GAA AAC CTC CTC AAG AAC CTG GAC CGG GTA   975
 G  V  K  G  I  G  E  K  T  A  L  K  L  L  K  E  W  G  S  L  E  N  L  L  K  N  L  D  R  V   225

AAG CCA GAA AAC GTC CGG GAG AAG ATC AAG GCC CAC CTG GAA GAC CTC AGG CTT TCC TTG GAG CTC TCC CGG GTG CGC ACC GAC CTC CCC  1065
 K  P  E  N  V  R  E  K  I  K  A  H  L  E  D  L  R  L  S  L  E  L  S  R  V  R  T  D  L  P   255

CTG GAG GTG GAC CTC GCC CAG GGG CGG GAG CCC GAC CGG GAG GGG CTT AGG GCC TTC CTG GAG AGG CTG GAG TTC GGC AGC CTC CTC CAC  1155
 L  E  V  D  L  A  Q  G  R  E  P  D  R  E  G  L  R  A  F  L  E  R  L  E  F  G  S  L  L  H   285

GAG TTC GGC CTC CTG GAG GCC CCC GCC CCC CTG GAG GAG GCC CCC TGG CCC CCG CCG GAA GGG GCC TTC GTG GGC TTC GTC CTC TCC CGC  1245
 E  F  G  L  L  E  A  P  A  P  L  E  E  A  P  W  P  P  P  E  G  A  F  V  G  F  V  L  S  R   315

CCC GAG CCC ATG TGG GCG GAG CTT AAA GCC CTG GCC GCC TGC AGG GAC GGC CGG GTG CAC CGG GCA GCA GAC CCC TTG GCG GGG CTA AAG  1335
 P  E  P  M  W  A  E  L  K  A  L  A  A  C  R  D  G  R  V  H  R  A  A  D  P  L  A  G  L  K   345
```

(Continued)

Figure 2 (i)

(Continued)

```
GAC CTC AAG GAG GTC CGG GGT CTC CTC GCC AAG GAC CTC GCC GTC TTG GCC TCG AGG GAG GGG CTA GAC CTC GTG CCC GGG GAC GAC CCC   1425
 D  L  K  E  V  R  G  L  L  A  K  D  L  A  V  L  A  S  R  E  G  L  D  L  V  P  G  D  D  P    375

ATG CTC CTC GCC TAC CTC CTG GAC CCC TCC AAC ACC ACC CCC GAG GGG GTG GCG CGG CGC TAC GGG GGG GAG TGG ACG GAG GAC GCC GCC   1515
 M  L  L  A  Y  L  L  D  P  S  N  T  T  P  E  G  V  A  R  R  Y  G  G  E  W  T  E  D  A  A    405

CAC CGG GCC CTC CTC TCG GAG AGG CTC CAT CGG AAC CTC CTT AAG CGC CTC GAG GGG GAG GAG AAG CTC CTT TGG CTC TAC CAC GAG GTG   1605
 H  R  A  L  L  S  E  R  L  H  R  N  L  L  K  R  L  E  G  E  E  K  L  L  W  L  Y  H  E  V    435

GAA AAG CCC CTC TCC CGG GTC CTG GCC CAC ATG GAG GCC ACC GGG GTA CGG CTG GAC GTG GCC TAC CTG CAG GCC CTT TCC CTG GAG CTT   1695
 E  K  P  L  S  R  V  L  A  H  M  E  A  T  G  V  R  L  D  V  A  Y  L  Q  A  L  S  L  E  L    465

GCG GAG GAG ATC CGC CGC CTC GAG GAG GAG GTC TTC CGC TTG GCG GGC CAC CCC TTC AAC CTC AAC TCC CGG GAC CAG CTG GAA AGG GTG   1785
 A  E  E  I  R  R  L  E  E  E  V  F  R  L  A  G  H  P  F  N  L  N  S  R  D  Q  L  E  R  V    495

CTC TTT GAC GAG CTT AGG CTT CCC GCC TTG GGG AAG ACG CAA AAG ACG GGC AAG CGC TCC ACC AGC GCC GCG GTG CTG GAG GCC CTA CGG   1875
 L  F  D  E  L  R  L  P  A  L  G  K  T  Q  K  T  G  K  R  S  T  S  A  A  V  L  E  A  L  R    525

GAG GCC CAC CCC ATC GTG GAG AAG ATC CTC CAG CAC CGG GAG CTC ACC AAG CTC AAG AAC ACC TAC GTG GAC CCC CTC CCA AGC CTC GTC   1965
 E  A  H  P  I  V  E  K  I  L  Q  H  R  E  L  T  K  L  K  N  T  Y  V  D  P  L  P  S  L  V    555

CAC CCG AGG ACG GGC CGC CTC CAC ACC CGC TTC AAC CAG ACG GCC ACG GCC ACG GGG AGG CTT AGT AGC TCC GAC CCC AAC CTG CAG AAC   2055
 H  P  R  T  G  R  L  H  T  R  F  N  Q  T  A  T  A  T  G  R  L  S  S  S  D  P  N  L  Q  N    585

ATC CCC GTC CGC ACC CCC TTG GGC CAG AGG ATC CGC CGG GCC TTC GTG GCC GAG GCG GGA TGG GCG TTG GTG GCC CTG GAC TAT AGC CAG   2145
 I  P  V  R  T  P  L  G  Q  R  I  R  R  A  F  V  A  E  A  G  W  A  L  V  A  L  D  Y  S  Q    615

ATA GAG CTC CGC GTC CTC GCC CAC CTC TCC GGG GAC GAG AAC CTG ATC AGG GTC TTC CAG GAG GGG AAG GAC ATC CAC ACC CAG ACC GCA   2235
 I  E  L  R  V  L  A  H  L  S  G  D  E  N  L  I  R  V  F  Q  E  G  K  D  I  H  T  Q  T  A    645

AGC TGG ATG TTC GGC GTC CCC CCG GAG GCC GTG GAC CCC CTG ATG CGC CGG GCG GCC AAG ACG GTG AAC TTC GGC GTC CTC TAC GGC ATG   2325
 S  W  M  F  G  V  P  P  E  A  V  D  P  L  M  R  R  A  A  K  T  V  N  F  G  V  L  Y  G  M    675

TCC GCC CAT AGG CTC TCC CAG GAG CTT GCC ATC CCC TAC GAG GAG GCG GTG GCC TTT ATA GAG CGC TAC TTC CAA AGC TTC CCC AAG GTG   2415
 S  A  H  R  L  S  Q  E  L  A  I  P  Y  E  E  A  V  A  F  I  E  R  Y  F  Q  S  F  P  K  V    705
```

(Continued)

Figure 2 (ii)

(Continued)

```
CGG GCC TGG ATA GAA AAG ACC CTG GAG GAG GGG AGG AAG CGG GGC TAC GTG GAA ACC CTC TTC GGA AGA AGG CGC TAC GTG CCC GAC CTC   2505
 R  A  W  I  E  K  T  L  E  E  G  R  K  R  G  Y  V  E  T  L  F  G  R  R  R  Y  V  P  D  L                              735

AAC GCC CGG GTG AAG AGC GTC AGG GAG GCC GCG GAG CGC ATG GCC TTC AAC ATG CCC GTC CAG GGC ACC GCC GCC GAC CTC ATG AAG CTC   2595
 N  A  R  V  K  S  V  R  E  A  A  E  R  M  A  F  N  M  P  V  Q  G  T  A  A  D  L  M  K  L                              765

GCC ATG GTG AAG CTC TTC CCC CGC CTC CGG GAG ATG GGG GCC CGC ATG CTC CTC CAG GTC CAC GAC GAG CTC CTC CTG GAG GCC CCC CAA   2685
 A  M  V  K  L  F  P  R  L  R  E  M  G  A  R  M  L  L  Q  V  H  D  E  L  L  L  E  A  P  Q                              795

GCG CGG GCC GAG GAG GTG GCG GCT TTG GCC AAG GAG GCC ATG GAG AAG GCC TAT CCC CTC GCC GTG CCC CTG GAG GTG GAG GTG GGG ATG   2775
 A  R  A  E  E  V  A  A  L  A  K  E  A  M  E  K  A  Y  P  L  A  V  P  L  E  V  E  V  G  M                              825

GGG GAG GAC TGG CTT TCC GCC AAG GGT TAGGGGGGCCCTGCCGTTTAGAGGAAGTTCAAGGGGTTGTCCCTCAGAAACGCCTCCAGGGGAACGCCCTCTGCGGCTACCAGG   2886
 G  E  D  W  L  S  A  K  G  *                                                                                              835

AGGCCTTTAGCCCCAAAGGTGCGGGTGAAGGCTTCCAGGCCCTGGGTTCTTTTAAAGGGGGCGCTTTTGACCTCGAGGGCCAGGAGGCGCTTTCCCTTTTGAAGGACAAAGTCACTTCCT   3006

GGTCCCTTTCCCGCCAGTAGTACACCTCAAACCCCCCCTGGT                                                                                3048
```

Figure 2 (iii)

```
                10        20        30        40        50        60        70        80
MBR TFL MEAIVPLFEPKGRVLLVDGHHLAYRTFFALKGLTTSRGEPVQAVYGFAKSLLKALKEDGYKAVFVVFDAKAPSFRHEAYE
TAQ     MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDG-DAVIVVFDAKAPSFRHEAYG
A&V TFL  MAMLPLFEPKGRVLLVDGHHLAYRTFFALKGLTTSRGEPVQAVYGFAKSLLKALKEDG-DVVVVVFDAKAPSFRHEAYE
         *^****                   ^                                ^** *               *

MBR TFL AYKAGRAPTPEDFPRQLALIKELVDLLGFTRLEVPGYEADDVLATLAKKAEKEGYEVRILTADRDLYQLVSDRVVVLHPE
TAQ     GYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLSDRIHVLHPE
A&V TFL AYKAGRAPTPEDFPRQLALIKELVDLLGLVRLEVPGFEADDVLATLAKRAEKEGYEVRILTADRDLYQLLSERIAILHPE
         *                        **      *       *   *               *     * * *^*

MBR TFL GHLITPEWLWEKYGLKPEQWVDFRALVGDPSDNLPGVKGIGEKTALKLLKEWGSLENLLKNLDRVKPENVREKIKAHLED
TAQ     GYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLKP-AIREKILAHMDD
A&V TFL GYLITPAWLYEKYGLRPEQWVDYRALAGDPSDNIPGVKGIGEKTAQRLIREWGSLENLFQHLDQVKP-SLREKLQAGMEA
         *   *        * *  * *   *  ^   *            ^* *^     * ***  **  ^**   *^ ^***

MBR TFL LRLSLELSRVRTDLPLEVDLAQGREPDREGLRAFLERLEFGSLLHEFGLLEAPAPLEEAPWPPPEGAFVGFVLSRPEPMW
TAQ     LKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMW
A&V TFL LALSRKLSQVHTDLPLEVDFGRRRTPNLEGLRAFLERLEFGSLLHEFGLLEGPKAAEEAPWPPPEGAFLGFSFSRPEPMW
         ^  ^* ** *       ***^ * *^ ^                  * ^*^           *  ^*  ^

MBR TFL AELKALAACRDGRVHRAADPLAGLKDLKEVRGLLAKDLAVLASREGLDLVPGDDPMLLAYLLDPSNTTPEGVARRYGGEW
TAQ     ADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEW
A&V TFL AELLALAGAWEGRLHRAQDPLRGLRDLKGVRGILAKDLAVLALREGLDLFPEDDPMLLAYLLDPSNTTPEGVARRYGGEW
         * ^   *^**  *   ** ^^* *   **  *     *   ^    * ^ *

MBR TFL TEDAAHRALLSERLHRNLLKRLEGEEKLLWLYHEVEKPLSRVLAHMEATGVRLDVAYLQALSLELAEEIRRLEEEVFRLA
TAQ     TEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLA
A&V TFL TEDAGERALLAERLFQTLKERLKGEERLLWLYEEVEKPLSRVLARMEATGVRLDVAYLQALSLEVEAEVRQLEEEVFRLA
         * **  ^ *   ^^* ^^  *   *     ^   *   ^   *             *     *** *^*  *

MBR TFL GHPFNLNSRDQLERVLFDELRLPALGKTQKTGKRSTSAAVLEALREAHPIVEKILQHRELTKLKNTYVDPLPSLVHPRTG
TAQ     GHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTG
A&V TFL GHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVDRILQYRELTKLKNTYIDPLPALVHPKTG
                         ^   *   *                    **   *       *   *    * *  *
```

Figure 3 (i)

(Continued)

(Continued)

```
MBR TFL RLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFVASGGWALVALDYSQIELRVLAHLSGDENLIRVFQEGKDI
TAQ     RLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDI
A&V TFL RLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFVAEEGWVLVVLDYSQIELRVLAHLSGDENLIRVFQEGRDI
                                                  * **  ^  *                              *

MBR TFL HTQTASWMFGVPPEAVDPLMRRAAKTVNFGVLYGMSAHRLSQELAIPYEEAVAFIERYFQSFPKVRAWIEKTLEEGRKRG
TAQ     HTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRG
A&V TFL HTQTASWMFGVSPEGVDPLMRRAAKTINFGVLYGMSAHRLSGELSIPYEEAVAFIERYFQSYPKVRAWIEGTLEEGRRRG
          *        *  *           *                ^  *     ^         *         ^       *

MBR TFL YVETLFGRRRYVPDLNARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLREMGARMLLQVHDELLLEAPQARAEE
TAQ     YVETLFGRRRYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQVHDELVLEAPKERAEA
A&V TFL YVETLFGRRRYVPDLNARVKSVREAAERMAFNMPVQGTAADLMKLAMVRLFPRLQELGARMLLQVHDELVLEAPKDRAER
                        *                                  *    ^ *             *    **   ^

MBR TFL VAALAKEAMEKAYPLAVPLEVEVGMGEDWLSAKG
TAQ     VARLAKEVMEGVYPLAVPLEVEVGIGEDWLSAKE
A&V TFL VAALAKEVMEGVWPLQVPLEVEVGLGEDWLSAKE
          ^    *  ^**  *        *        *
```

* = Conservative change ^ = Non-conservative change

Figure 3 (ii)

```
        10          20          30          40          50          60
tacttcggcg gggtgaagct cggggccggg gggcttgtgc gggcctacgg gggggtggcg
atgaagccgc cccacttcga gccccggccc cccgaacacg cccggatgcc cccccaccgc

        70          80          90         100         110 FTFLZ   120
gcggaggcct taagcgggcg cccaaggtcc ccttggtgga gcgggtgggg ctcgccttcc
cgcctccgga attcgcccgc gggttccagg ggaaccacct cgcccacccc gagcggaagg

       130         140         150         160         170         180
tcgtgccctt cgccgaggtg ggccgggtct acgccctcct ggaggcccgc gccctgaagg
agcacgggaa gcggctccac ccggcccaga tgcgggagga cctccgggcg cgggacttcc

       190         200         210         220         230         240
ccgaggagac ctacaccccg gagggcgtgc gcttcgccct cctcctcccc aagcccgagc
ggctcctctg gatgtggggc ctcccgcacg cgaagcggga ggaggagggg ttcgggctcg

       250 FTFLQ  260         270         280         290 FTFL10  300
gggaaggttt cctcagggcg ctcctggacg ccacccgggg acaggtggcc ctggagtagc
ccctttccaa ggagtcccgc gaggacctgc ggtgggcccc tgtccaccgg gacctcatcg

          RTFLP
       310         320 FTFLX   330         340         350         360
ATGGAGGCGA TCGTTCCGCT CTTTGAACCC AAAGGCCGGG TCCTCCTGGT GGACGGCCAC
TACCTCCGCT AGCAAGGCGA GAAACTTGGG TTTCCGGCCC AGGAGGACCA CCTGCCGGTG
                    RTFLW
       370         380         390         400         410         420
CACCTGGCCT ACCGCACCTT CTTCGCCCTG AAGGGCCTCA CCACGAGCCG GGGCGAACCG
GTGGACCGGA TGGCGTGGAA GAAGCGGGAC TTCCCGGAGT GGTGCTCGGC CCCGCTTGGC

       430         440         450         460         470         480
GTGCAGGCGG TCTACGGCTT CGCCAAGAGC CTCCTCAAGG CCCTGAAGGA GGACGGGTAC
CACGTCCGCC AGATGCCGAA GCGGTTCTCG GAGGAGTTCC GGGACTTCCT CCTGCCCATG
                                            RTFLY
FTFLL  490         500         510         520         530         540
AAGGCCGTCT TCGTGGTCTT TGACGCCAAG GCCCCCTCCT TCCGCCACGA GGCCTACGAG
TTCCGGCAGA AGCACCAGAA ACTGCGGTTC CGGGGGAGGA AGGCGGTGCT CCGGATGCTC
RTFLK
       550         560         570         580         590         600
GCCTACAAGG CGGGGAGGGC CCCGACCCCC GAGGACTTCC CCCGGCAGCT CGCCCTCATC
CGGATGTTCC GCCCCTCCCG GGGCTGGGGG CTCCTGAAGG GGGCCGTCGA GCGGGAGTAG

       610         620         630         640         650         660
AAGGAGCTGG TGGACCTCCT GGGGTTTACC CGCCTCGAGG TCCCCGGCTA CGAGGCGGAC
TTCCTCGACC ACCTGGAGGA CCCCAAATGG GCGGAGCTCC AGGGGCCGAT GCTCCGCCTG
```

(Continued)

Figure 4 (i)

(Continued)

```
      670         680         690         700         710         720
GACGTCCTCG CCACCCTGGC CAAGAAGGCG GAAAAGGAGG GGTACGAGGT GCGCATCCTC
CTGCAGGAGC GGTGGGACCG GTTCTTCCGC CTTTTCCTCC CCATGCTCCA CGCGTAGGAG

      730 FTFLB  740         750         760         770         780
ACCGCCGACC GCGACCTCTA CCAACTCGTC TCCGACCGCG TCGTCGTCCT CCACCCCGAG
TGGCGGCTGG CGCTGGAGAT GGTTGAGCAG AGGCTGGCGC AGCAGCAGGA GGTGGGGCTC
           RTFLA
      790         800         810         820         830         840
GGCCACCTCA TCACCCCGGA GTGGCTTTGG GAGAAGTACG GCCTCAAGCC GGAGCAGTGG
CCGGTGGAGT AGTGGGGCCT CACCGAAACC CTCTTCATGC CGGAGTTCGG CCTCGTCACC

      850         860         870         880         890         900
GTGGACTTCC GCGCCCTCGT GGGGGACCCC TCCGACAACC TCCCCGGGGT CAAGGGCATC
CACCTGAAGG CGCGGGAGCA CCCCCTGGGG AGGCTGTTGG AGGGGCCCCA GTTCCCGTAG

      910         920         930 FTFLM  940         950         960
GGGGAGAAGA CCGCCCTCAA GCTCCTCAAG GAGTGGGGAA GCCTGGAAAA CCTCCTCAAG
CCCCTCTTCT GGCGGGAGTT CGAGGAGTTC CTCACCCCTT CGGACCTTTT GGAGGAGTTC
                                  RTFLN
      970         980         990        1000        1010        1020
AACCTGGACC GGGTAAAGCC AGAAAACGTC CGGGAGAAGA TCAAGGCCCA CCTGGAAGAC
TTGGACCTGG CCCATTTCGG TCTTTTGCAG GCCCTCTTCT AGTTCCGGGT GGACCTTCTG

     1030 FTFL15 1040        1050        1060        1070        1080
CTCAGGCTTT CCTTGGAGCT CTCCCGGGTG CGCACCGACC TCCCCCTGGA GGTGGACCTC
GAGTCCGAAA GGAACCTCGA GAGGGCCCAC GCGTGGCTGG AGGGGGACCT CCACCTGGAG
TFLER1      RTFL16
     1090        1100        1110        1120        1130 TFLEF1 1140
GCCCAGGGGC GGGAGCCCGA CCGGGAGGGG CTTAGGGCCT TCCTGGAGAG GCTGGAGTTC
CGGGTCCCCG CCCTCGGGCT GGCCCTCCCC GAATCCCGGA AGGACCTCTC CGACCTCAAG

     1150        1160        1170        1180        1190        1200
GGCAGCCTCC TCCACGAGTT CGGCCTCCTG GAGGCCCCCG CCCCCCTGGA GGAGGCCCCC
CCGTCGGAGG AGGTGCTCAA GCCGGAGGAC CTCCGGGGGC GGGGGGACCT CCTCCGGGGG

     1210        1220        1230 FTFLR 1240        1250        1260
TGGCCCCCGC CGGAAGGGGC CTTCGTGGGC TTCGTCCTCT CCCGCCCCGA GCCCATGTGG
ACCGGGGGCG GCCTTCCCCG GAAGCACCCG AAGCAGGAGA GGGCGGGGCT CGGGTACACC
                       RTFLC
     1270        1280        1290        1300        1310        1320
GCGGAGCTTA AAGCCCTGGC CGCCTGCAGG GACGGCCGGG TGCACCGGGC AGCAGACCCC
CGCCTCGAAT TTCGGGACCG GCGGACGTCC CTGCCGGCCC ACGTGGCCCG TCGTCTGGGG
```

(Continued)

Figure 4 (ii)

(Continued)

```
      1330        1340        1350        1360        1370        1380
TTGGCGGGGC TAAAGGACCT CAAGGAGGTC CGGGGTCTCC TCGCCAAGGA CCTCGCCGTC
AACCGCCCCG ATTTCCTGGA GTTCCTCCAG GCCCCAGAGG AGCGGTTCCT GGAGCGGCAG

      1390        1400        1410        1420        1430 FTFLE 1440
TTGGCCTCGA GGGAGGGGCT AGACCTCGTG CCCGGGGACG ACCCCATGCT CCTCGCCTAC
AACCGGAGCT CCCTCCCCGA TCTGGAGCAC GGGCCCCTGC TGGGGTACGA GGAGCGGATG
                                                RTFLD

      1450        1460        1470        1480        1490        1500
CTCCTGGACC CCTCCAACAC CACCCCCGAG GGGGTGGCGC GGCGCTACGG GGGGGAGTGG
GAGGACCTGG GGAGGTTGTG GTGGGGGCTC CCCCACCGCG CCGCGATGCC CCCCCTCACC

      1510        1520        1530        1540        1550        1560
ACGGAGGACG CCGCCCACCG GGCCCTCCTC TCGGAGAGGC TCCATCGGAA CCTCCTTAAG
TGCCTCCTGC GGCGGGTGGC CCGGGAGGAG AGCCTCTCCG AGGTAGCCTT GGAGGAATTC

      1570        1580        1590        1600        1610        1620
CGCCTCGAGG GGGAGGAGAA GCTCCTTTGG CTCTACCACG AGGTGGAAAA GCCCCTCTCC
GCGGAGCTCC CCCTCCTCTT CGAGGAAACC GAGATGGTGC TCCACCTTTT CGGGGAGAGG

      1630        1640        1650        1660        1670        1680
CGGGTCCTGG CCCACATGGA GGCCACCGGG GTACGGCTGG ACGTGGCCTA CCTGCAGGCC
GCCCAGGACC GGGTGTACCT CCGGTGGCCC CATGCCGACC TGCACCGGAT GGACGTCCGG

      1690        1700        1710        1720 FTFLF 1730        1740
CTTTCCCTGG AGCTTGCGGA GGAGATCCGC CGCCTCGAGG AGGAGGTCTT CCGCTTGGCG
GAAAGGGACC TCGAACGCCT CCTCTAGGCG GCGGAGCTCC TCCTCCAGAA GGCGAACCGC
                                    RTFL18A

      1750        1760        1770        1780        1790        1800
GGCCACCCCT TCAACCTCAA CTCCCGGGAC CAGCTGGAAA GGGTGCTCTT TGACGAGCTT
CCGGTGGGGA AGTTGGAGTT GAGGGCCCTG GTCGACCTTT CCCACGAGAA ACTGCTCGAA

      1810        1820        1830        1840        1850        1860
AGGCTTCCCG CCTTGGGGAA GACGCAAAAG ACGGGCAAGC GCTCCACCAG CGCCGCGGTG
TCCGAAGGGC GGAACCCCTT CTGCGTTTTC TGCCCGTTCG CGAGGTGGTC GCGGCGCCAC

      1870        1880        1890        1900 FTFL17 1910        1920
CTGGAGGCCC TACGGGAGGC CCACCCCATC GTGGAGAAGA TCCTCCAGCA CCGGGAGCTC
GACCTCCGGG ATGCCCTCCG GGTGGGGTAG CACCTCTTCT AGGAGGTCGT GGCCCTCGAG
                                    RTFL18

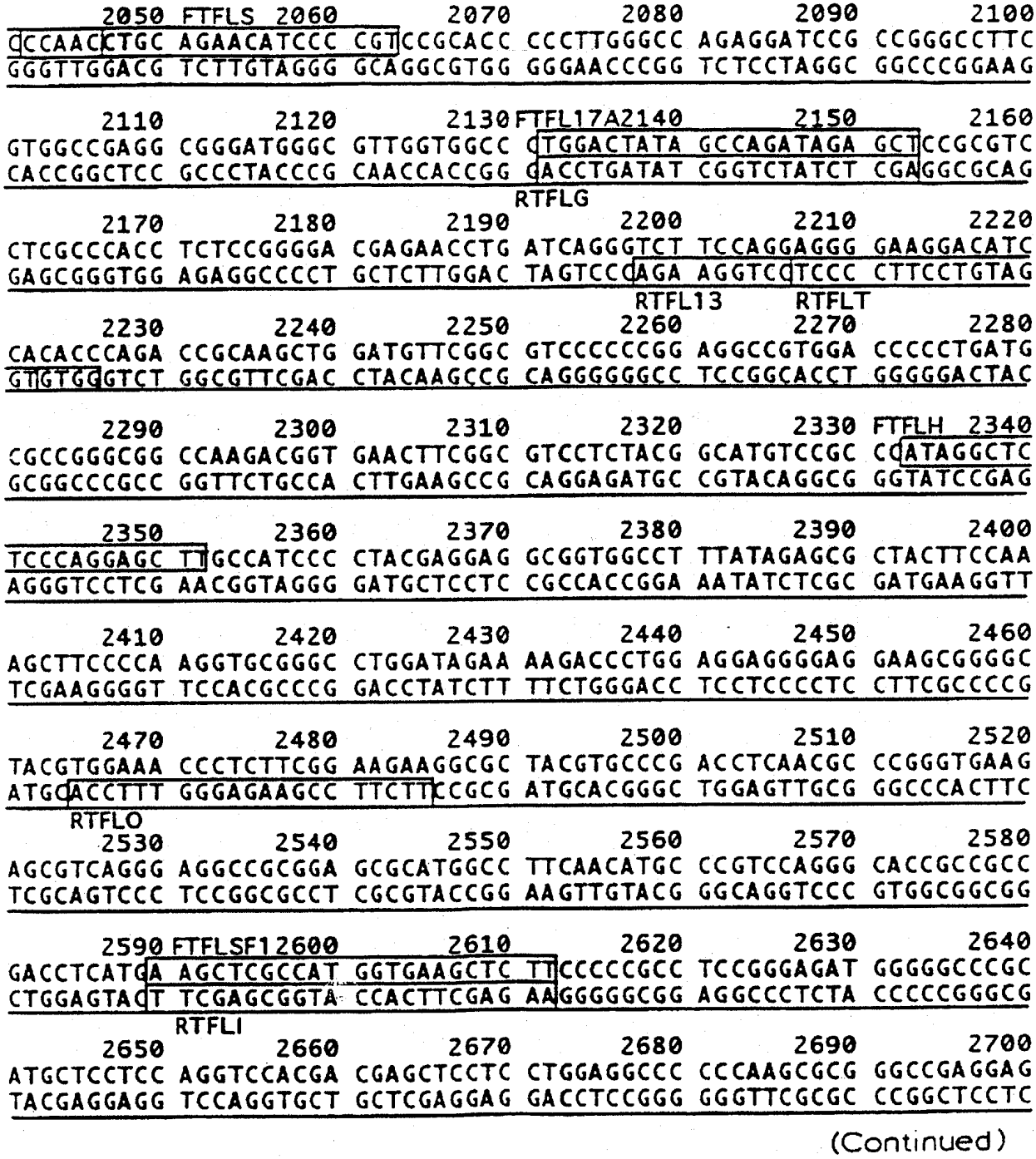
      1930        1940        1950        1960        1970        1980
ACCAAGCTCA AGAACACCTA CGTGGACCCC CTCCCAAGCC TCGTCCACCC GAGGACGGGC
TGGTTCGAGT TCTTGTGGAT GCACCTGGGG GAGGGTTCGG AGCAGGTGGG CTCCTGCCCG
                                                FTFL2

      1990        2000        2010        2020        2030 FTFL11 2040
CGCCTCCACA CCCGCTTCAA CCAGACGGCC ACGGCCACGG GGAGGCTTAG TAGCTCCGAC
GCGGAGGTGT GGGCGAAGTT GGTCTGCCGG TGCCGGTGCC CCTCCGAATC ATCGAGGCTG
```

(Continued)

Figure 4(iii)

(Continued)



(Continued)

Figure 4 (iv)

(Continued)

```
      2710        2720        2730        2740        2750        2760
GTGGCGGCTT TGGCCAAGGA GGCCATGGAG AAGGCCTATC CCCTCGCCGT GCCCCTGGAG
CACCGCCGAA ACCGGTTCCT CCGGTACCTC TTCCGGATAG GGGAGCGGCA CGGGGACCTC

      2770        2780        2790        2800        2810   FTFLU 2820
GTGGAGGTGG GGATGGGGGA GGACTGGCTT TCCGCCAAGG GTTAGgggag ccctgccgtt
CACCTCCACC CCTACCCCCT CCTGACCGAA AGGCGGTTCC CAATCccccc gggacggcaa
                                                    RTFLV
                          RTFL4/RTFL12
      2830        2840        2850        2860        2870        2880
tagaggaagt tcaagggttt gtccctcaga aacgcctcca ggggaacgcc ctctgcggct
atctccttca agttcccaaa caggg agtct ttgcggaggt ccccttgcgg gagacgccga
                          RTFLJ
      2890        2900        2910        2920        2930        2940
accaggaggc ctttagcccc aaaggtgcgg gtgaaggctt ccaggccctg ggttctttta
tggtcctccg gaaatcgggg tttccacgcc cacttccgaa ggtccgggac ccaagaaaat

      2950        2960        2970        2980        2990        3000
aaggggcgc ttttgacctc gagggccagg aggcgctttc ccttttgaag gacaaagtca
ttcccccgcg aaaactggag ctcccggtcc tccgcgaaag ggaaaacttc ctgtttcagt

      3010        3020        3030        3040        3050        3060
cttcctggtc cctttcccgc cagtagtaca cctcaaaccc cccctggt.. ..........
gaaggaccag ggaaagggcg gtcatcatgt ggagtttggg ggggacca.. ..........
```

Figure 4(v)

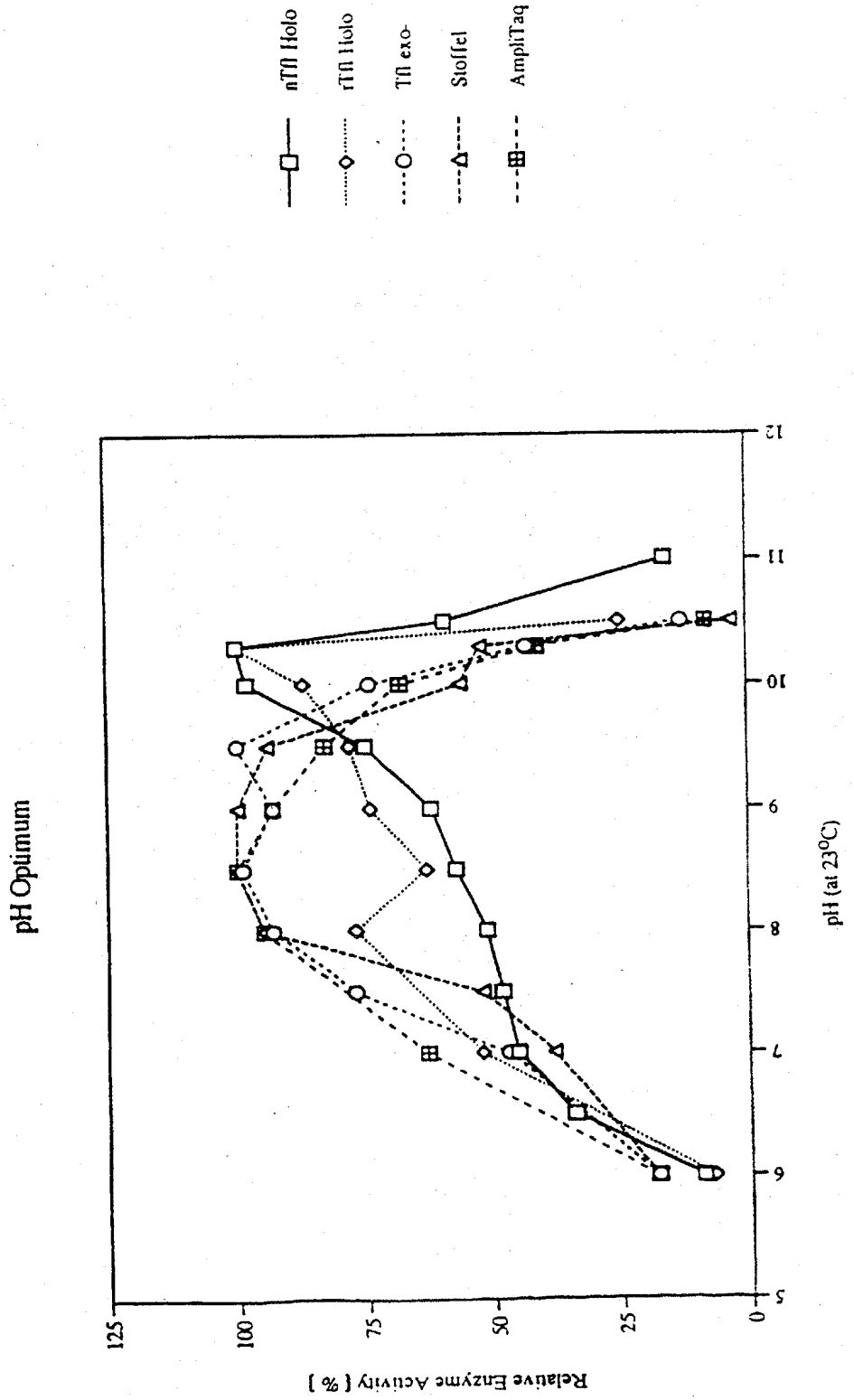

nTfl Holo
rTfl Holo
Tfl exo-
Stoffel
AmpliTaq
pH Optimum
Relative Enzyme Activity (%)
pH (at 23°C)
125
100
75
50
25
0
12
11
10
9
8
7
6
5

Figure 5

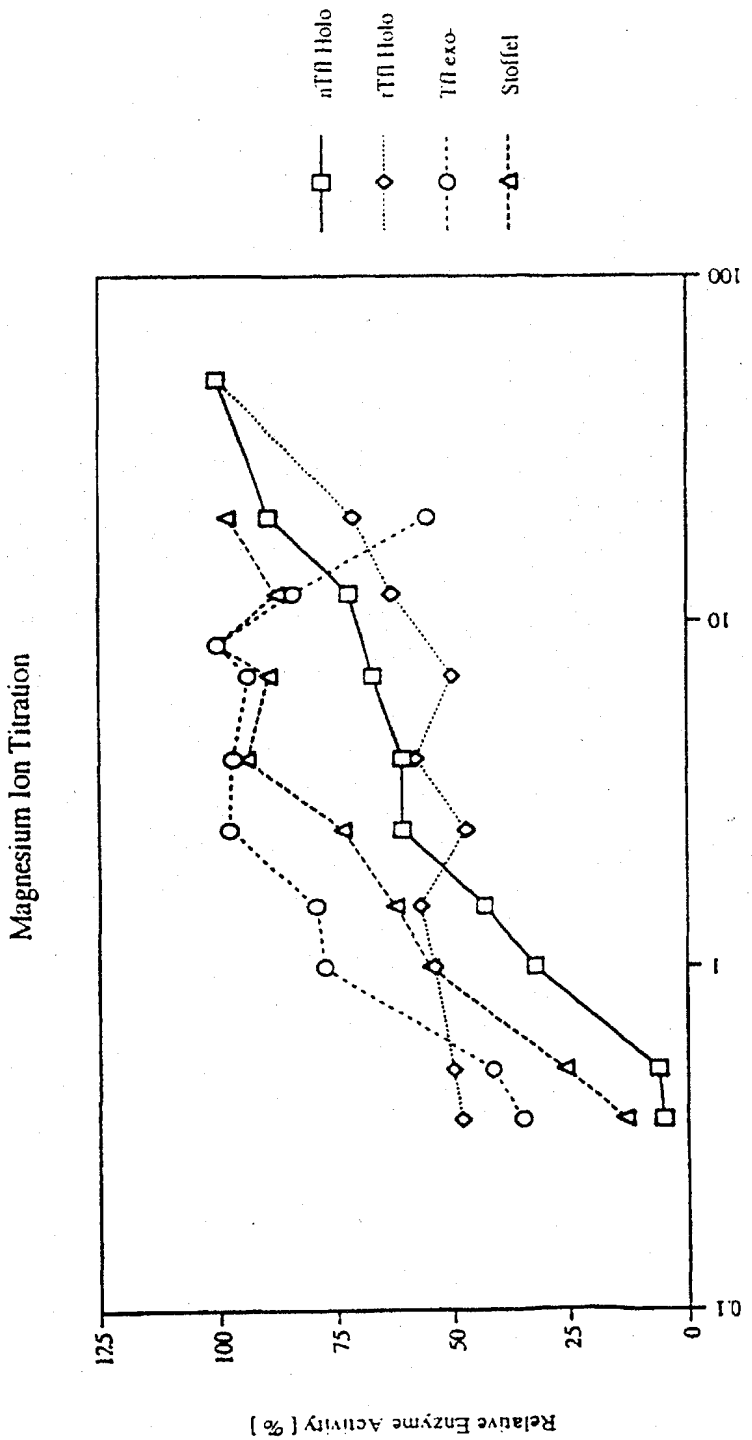

nTfl Holo
rTfl Holo
Tfl exo-
Stoffel
Magnesium Ion Titration
Relative Enzyme Activity [%]
125
100
75
50
25
0
0.1
1
10
100
MgCl2 [mM]

Figure 6A

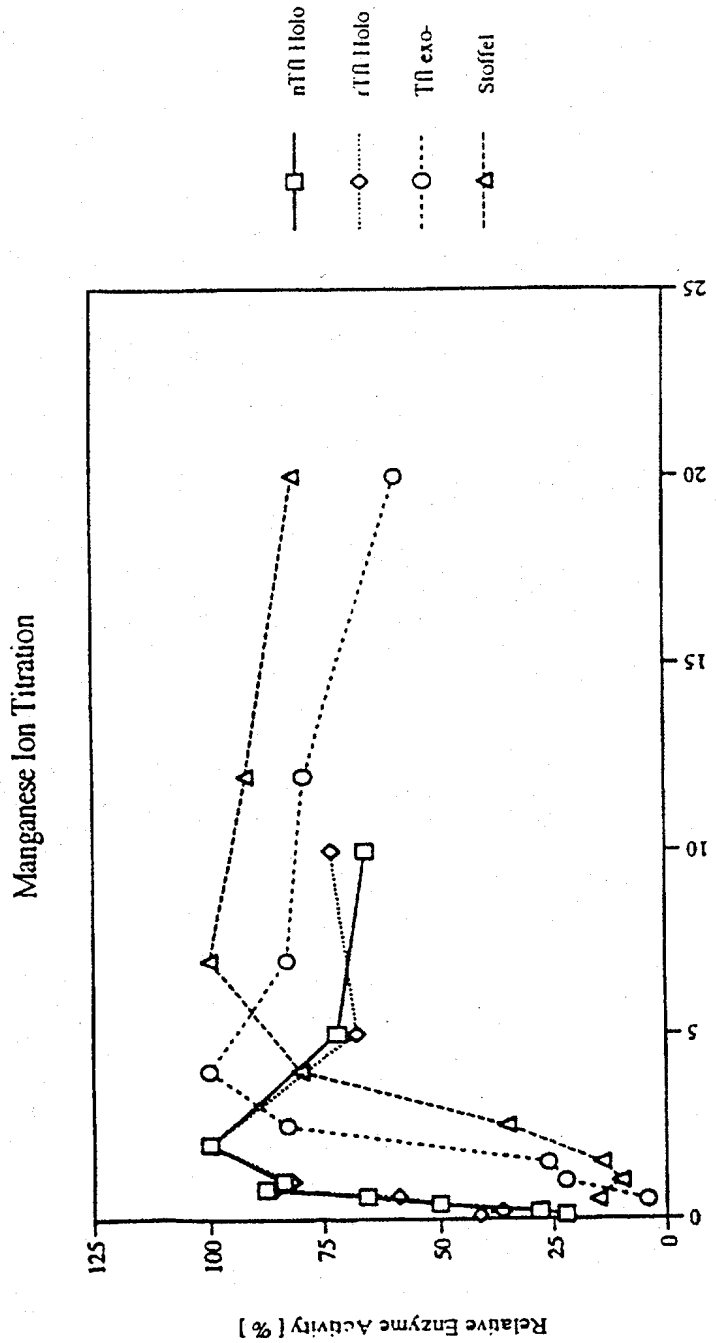

nTfl Holo
rTfl Holo
Tfl exo-
Stoffel
Manganese Ion Titration
Relative Enzyme Activity [%]
125
100
75
50
25
0
0
5
10
15
20
25
$MnCl_2$ [mM]

Figure 6B

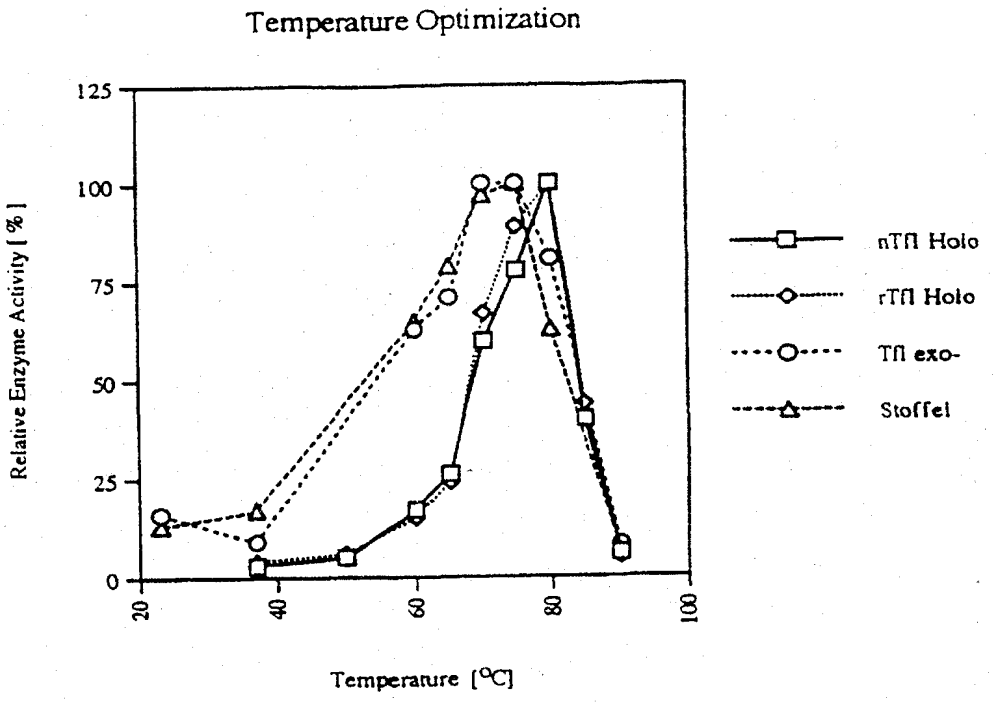
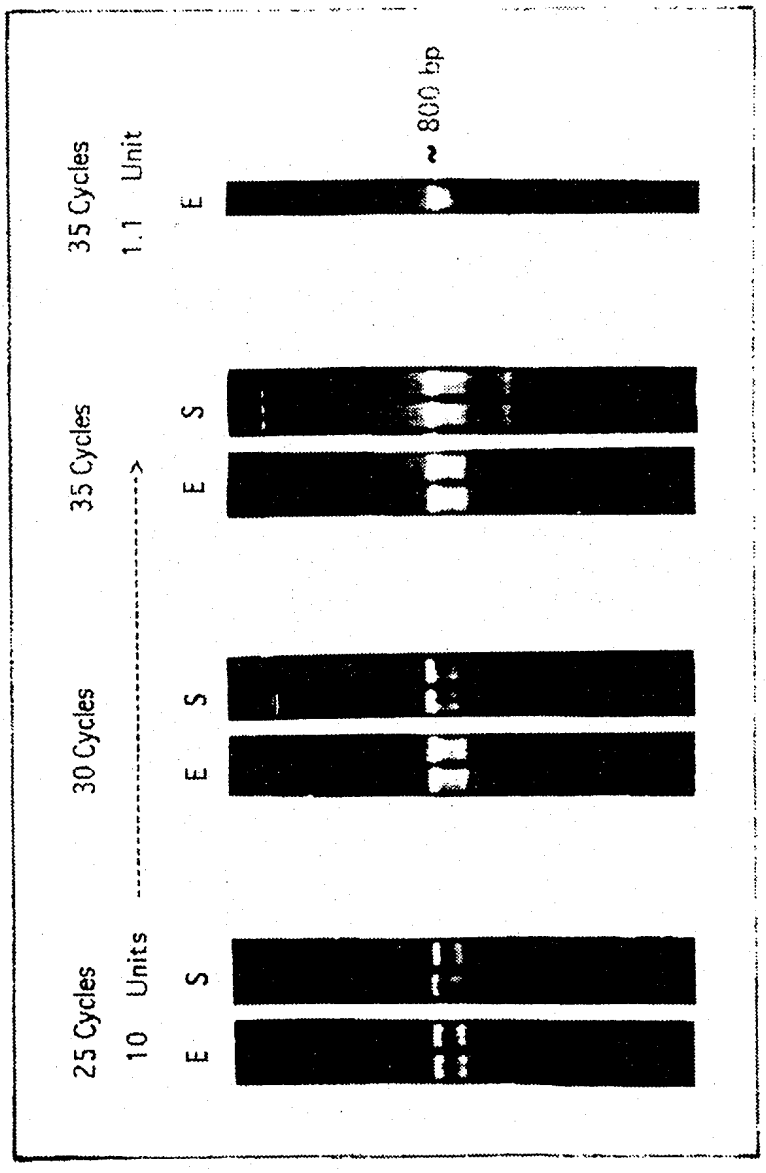

Temperature Optimization
Relative Enzyme Activity [%]
125
100
75
50
25
0
20
40
60
80
100
Temperature [°C]
nTfl Holo
rTfl Holo
Tfl exo-
Stoffel

Figure 7A

Figure 7B


~ 800 bp
25 Cycles
10 Units
30 Cycles
35 Cycles
1.1 Unit
35 Cycles
E
S
E
S
E
S
E

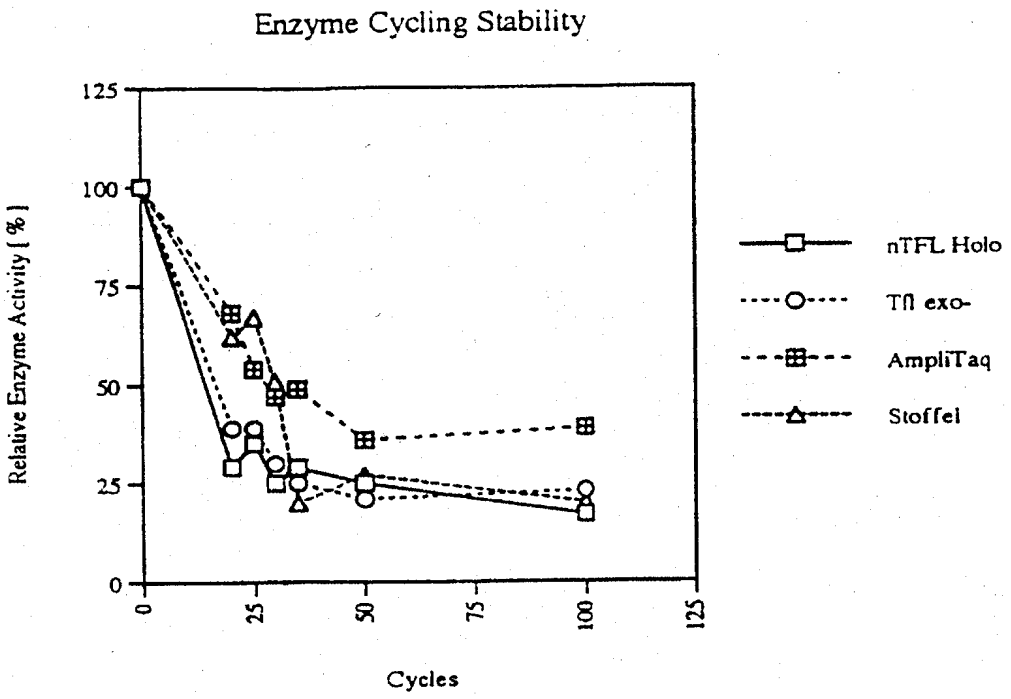

Enzyme Cycling Stability
Relative Enzyme Activity [%]
125
100
75
50
25
0
0
25
50
75
100
125
Cycles
nTFL Holo
Tfl exo-
AmpliTaq
Stoffel

Figure 8

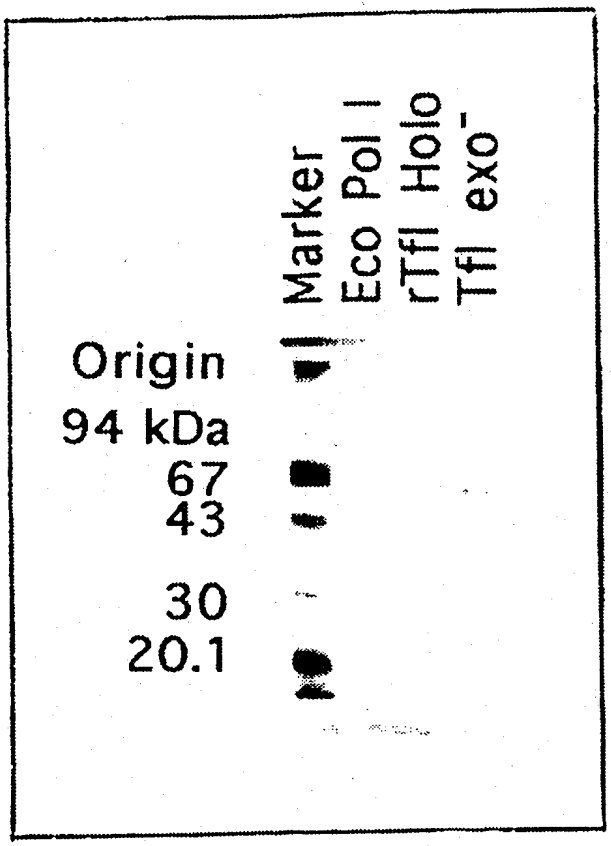


FIGURE 9

Figure 10A

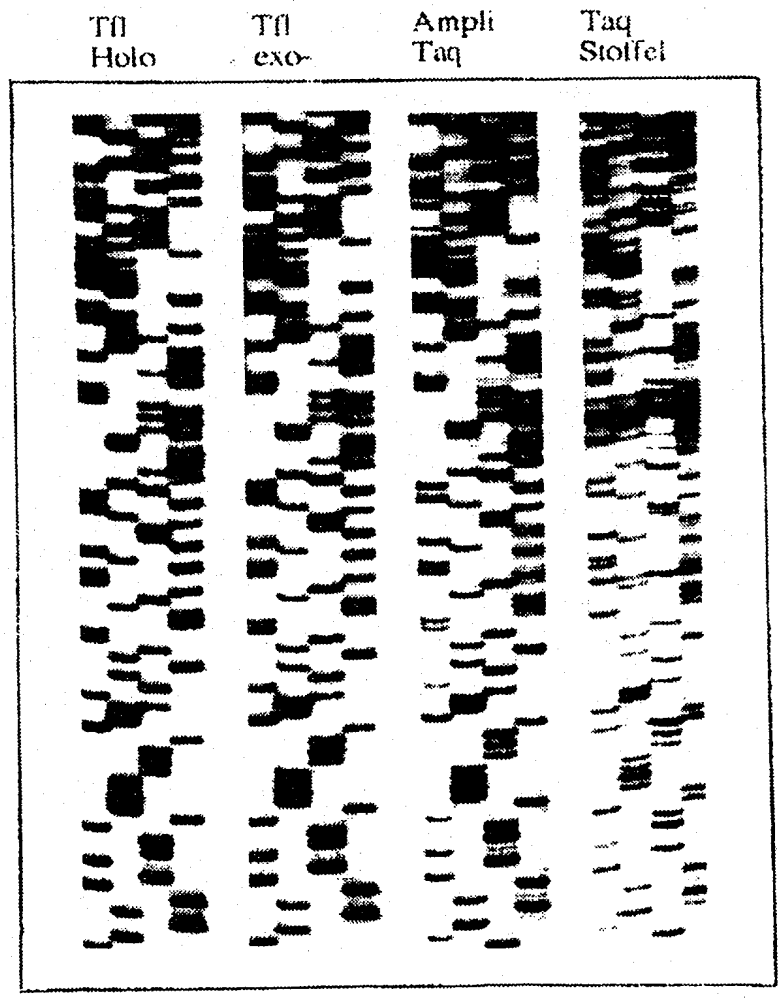


Figure 10B

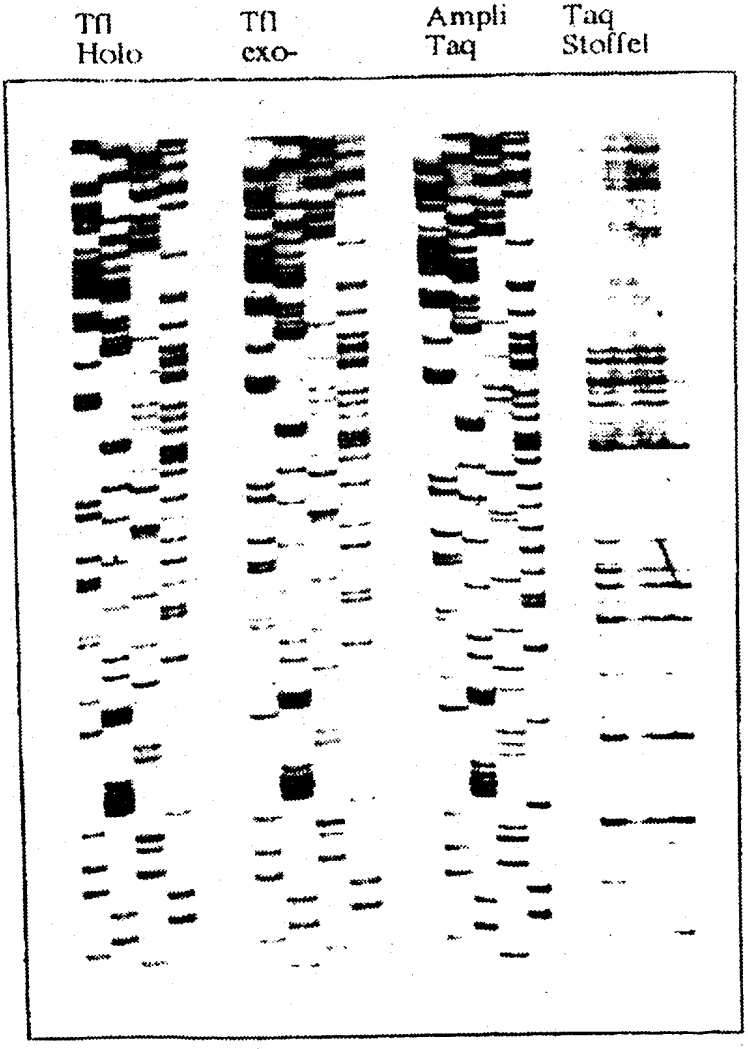


Figure 10C

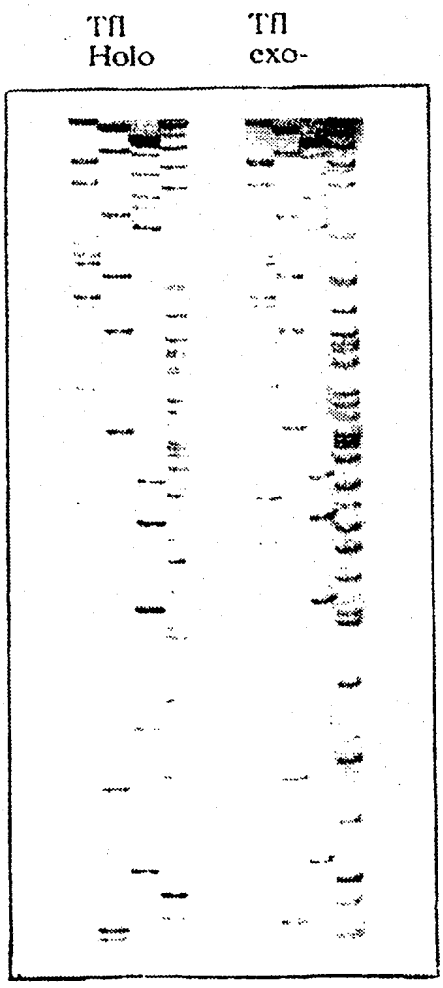
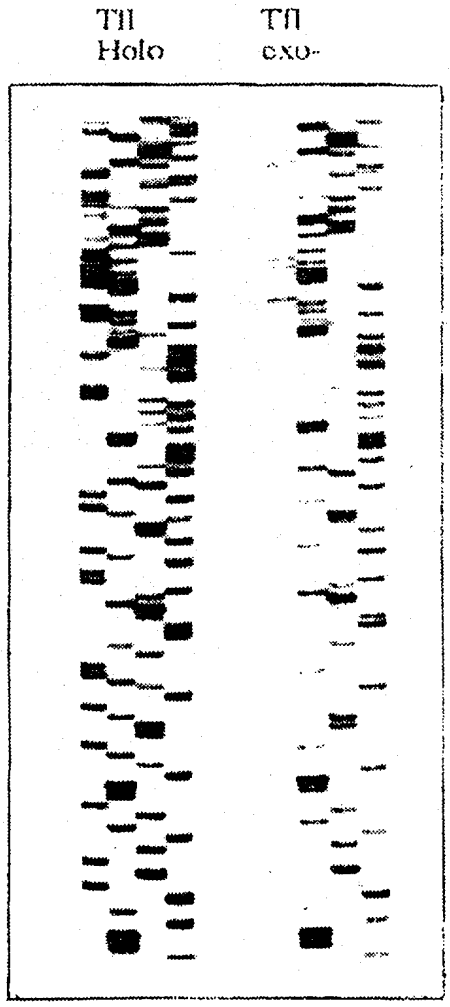


Figure 10D



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(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 80, line 1-10 (and elsewhere)

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American Type Culture Collection (ATCC)

Address of depositary institution *(including postal code and country)*

12301 Parklawn Dr.
Rockville, MD 20852
USA

Date of deposit	Accession Number
(1) 26 May 1994	(1) 69632
(2) 26 May 1994	(2) 69633

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Depositor: Molecular Biology Resources, Inc.
These deposits were made pursuant to the provisions of the Budapest Treaty
Host strains: (1) E coli DH5∝F'
(2) E coli DH5∝F'IQ

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C07K 14/195, C12N 1/21

A3

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08/334,645 4 November 1994 (04.11.94) US

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(74) Agents: POCHOPIEN, Donald, J. et al.; 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606-6402 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.
Date of receipt by the International Bureau:
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(88) Date of publication of the international search report:
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(54) Title: BIOLOGICALLY ACTIVE FRAGMENTS OF THERMUS FLAVUS DNA POLYMERASE

(57) Abstract

The present invention is directed to a DNA encoding a biologically active fragment of a thermostable, full length DNA polymerase I enzyme of *Thermus flavus*. More particularly, the invention is directed to a DNA encoding an approximately 63,000 dalton DNA polymerase that lacks 274 amino acids from the N-terminus of the approximately 94,000 dalton *T. flavus* DNA polymerase I, and to the protein encoded thereby which has been designated the *T. flavus* DNA polymerase I exo fragment. The enzyme fragments are useful in DNA sequencing, Thermal Cycle Labeling, Polymerase Chain Reaction, and other molecular biological applications.

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In ional Application No
PCT/US 95/15327

A. CLASSIFICATION OF SUBJECT MATTER
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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P,X	WO,A,94 29482 (THIRD WAVE TECHNOLOGIES, INC.) 22 December 1994 see page 9, line 18 - page 10, line 22 see page 29, line 20 - page 32, line 2 see SEQ ID NO:6 see figure 2; example 2 ---	1,6-9, 12-14,16
X Y	WO,A,91 09950 (CETUS CORPORATION) 11 July 1991 see page 2, line 18 - page 3, line 37 see page 5, line 20 - page 18, line 30 see page 22, line 3 - page 23, line 10 see examples 1,2,7 --- -/--	1,6-9, 12-14,16 2-5,10, 11,15, 17-25

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14 August 1996	0 3. 09. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	**Authorized officer** Donath, C

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